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DETERMINATION OF FREE CATECHOLAMINES IN URINE BY TANDEM AFFINITY/ION-PAIR CHROMATOGRAPHY AND FLOW INJECTION ANALYSIS

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Abstract

Background—A system was developed for the simultaneous measurement in urine of free catecholamines (i.e., dopamine, norepinephrine, epinephrine) and creatinine (i.e., an indicator of urine output and volume). This method was based on the use of tandem affinity/ion-pair HPLC and flow injection analysis.

Methods—The free catecholamines were extracted directly from urine by using an on-line phenylboronic acid affinity column. The extracted solutes were then separated and measured by ion-pair chromatography followed by amperometric detection. Creatinine was measured by an on-line flow injection analysis system based on the Jaffe reaction, which analyzed creatinine as it eluted non-retained from the phenylboronic acid column.

Results—Various factors were considered in the design and optimization of the phenylboronic acid column, the tandem affinity/ion-pair HPLC columns and the flow injection analysis system. The total analysis time for the final combined system was approximately 16 min per injection at 1 ml/min. This method was found to have good agreement with the expected results for control urine samples. The limits of detection for 20 μl samples (S/N = 3.0) were 1.8, 1.0 and 4.3 μg/l for norepinephrine, epinephrine and dopamine, respectively, while the limit of detection of creatinine was 5.0 mg/l. The linear response of this method extended over a 450 to 930-fold range in concentration for the catecholamines and covered the range of clinical interest. The within-day precision of this method was ±2.0−2.7%.

Conclusions—The ability of this method to simultaneously monitor both creatinine and other analytes makes this HPLC/FIA system an attractive method for use in monitoring urinary compounds. With this approach it was possible to provide fast results for small volumes of random urine samples that were collected as part of a psychological study. The same method could also be utilized with 12 or 24 h urine specimens.

Keywords

Free catecholamines; creatinine; affinity chromatography; phenylboronic acid; ion-pair HPLC; flow injection analysis

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1. Introduction

Affinity chromatography exploits the specific, yet reversible binding that occurs in many biological systems. In this technique, one of a pair of interacting compounds is immobilized onto a solid support and is used as a ligand for the selective separation or extraction of complementary molecules from a sample. Examples of ligands that can be employed in affinity chromatography include antibodies, triazine dyes, metal chelates, and phenylboronic acid, among many others [1]. When these affinity ligands are attached to an HPLC support such as silica, the resulting technique is known as high performance affinity chromatography (HPAC). One advantage of using affinity chromatography and HPAC is that the specificity of these techniques often eliminates the need for manual sample cleanup steps during the study of complex samples. This feature helps to reduce the time and labor need for the overall analysis and tends to increase the precision of the method, making these tools appealing for use in clinical analysis [2,3].

One disadvantage to the specificity of affinity chromatography is this also results in information being lost on compounds that are not retained by the immobilized ligand. Several approaches have been taken to recover this information by using mixed affinity supports, coupled columns and column switching methods [4-6]. An alternative way for expanding the range of chemicals that can be examined is to couple a flow injection analysis (FIA) system with the waste stream of an HPAC column [7]. This current report will examine the combined use of HPAC and FIA in the determination of unconjugated, or free, catecholamines in urine.

Catecholamines play an important role in neurotransmission and other physiological processes. Figure 1 shows the structures of the catecholamines which will be examined in this study, including dopamine, norepinephrine (noradrenaline) and epinephrine (adrenaline). These catecholamines are synthesized from tyrosine. Tyrosine undergoes hydroxylation and decarboxylation to produce dopamine, which is then hydroxylated and methylated to produce norepinephrine and epinephrine, respectively [8]. Related metabolites that are produced through the methylation or deamination of these catecholamines are 3,4-dihydroxymandelic acid, normetanephrine, metanephrine, 3-methoxythyramine, 3,4-dihydroxyphenylacetic acid, vanillymandelic acid, and homovanillic acid [8]. Information on the rate of catecholamine production, metabolism and excretion has been shown to be useful for psychological studies involving stress and its management [9]. Increased levels of certain catecholamines can be associated with hypertension, chromaffinoma, and some neural tumors (e.g., gangliomas or neuroblastomes); increased catecholamine levels have also been noted following heart failure [10].

Dopamine, norepinephrine and ephinephrine are routinely determined in clinical laboratories by reversed-phase liquid chromatography (RPLC) or ion-pair HPLC with electrochemical detection. This method is sensitive, but requires tedious and expensive sample pretreatment, including the use of liquid-liquid extraction, low-performance liquid chromatography [11] and solid phase extraction [12]. Automated on-line extractions using ion exchange or phenylboronic acid affinity supports have also been reported more recently [13]. This study will employ a modification of this latter technique by using ion-pair HPLC on a reversed-phase column and an on-line phenylboronic acid affinity column for the extraction and analysis of free catecholamines in urine samples.

One difficulty with analyzing compounds such as catecholamines in urine is related to the variable volume of urine that is excreted by different individuals or even by a single individual at different times. A correction can be made for this variability in urine output by comparing the concentration of each catecholamine in a urine sample to the concentration of another compound in the same sample that is excreted into the urine at a constant rate. Creatinine is

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the compound that is used most often for this purpose in clinical studies [10]. Creatinine is the anhydride of creatine and is a by-product of muscle metabolism. Creatinine is produced and excreted at a relatively constant rate by the body, making it a useful reference compound to correct for fluctuations in urine output and volume. The classical approach for measuring creatinine is the Jaffe method, which a colorimetric method based on formation of a highly colored product when creatinine reacts with an alkaline picrate solution. This technique may be performed as either a manual method or by flow injection analysis (FIA) [7,8,14].

We developed a fully automated system for measuring the concentration of creatinine in urine along with urinary free catecholamines. This system will couple the use of affinity extraction and ion-pair chromatography for the determination of catecholamines with an FIA method for the determination of creatinine. An affinity column containing acetylated phenylboronic acid silica will be used for the on-line extraction catecholamines from injected urine samples (see Ref. [16] for a recent review on phenylboronic acid columns and boronate affinity chromatography). The purified catecholamines will then be eluted, separated by ion-pair RPLC and detected electrochemically. Meanwhile, creatinine in the non-retained fraction of the affinity column will be determined on-line by using FIA with UV/Vis absorbance detection. Several items will be considered in the development of this method, including the performance and behavior of the HPAC column and FIA system as well as the final overall system.

2. Materials and Methods

2.1 Materials

The Nucleosil 100–7 silica (7 μm particle diameter, 100Å pore size) and C_{18} Nucleosil Si-100 –3 silica (3 μm particle diameter, 100 Å pore size) were from Alltech (Deerfield, IL). The 3-glycidoxypropyltrimethoxysilane, picric acid and creatinine were from Aldrich (Milwaukee, WI). HPLC-grade methanol and UriChem human urine controls were purchased from Fisher (Fair Lawn, NJ). The 3-aminophenylboronic acid, norepinephrine hydrochloride, epinephrine, dopamine hydrochloride, reduced glutathione (GSH), disodium ethylenediaminetetraacetic acid (Na_2EDTA) and sodium octylsulfate were from Sigma (St. Louis, MO). Other chemicals and biochemicals used in this study were of the purest grades available. All solutions were prepared using water from a NANOpure water system (Barnstead, Dubuque, IA).

2.2 Apparatus

A diagram of the HPLC/FIA system that was used in this study is shown in Figure 2. The chromatographic component of this system was comprised of 2 CM3000 or CM3200 pumps and a GM4000 gradient programmer (LDC/Milton Roy, Riviera Beach, FL). Samples were injected using an LDC/Milton Roy 715 autosampler equipped with a 7020 Rheodyne injection valve (Cotati, CA) and a 20 μl loop. The application and elution buffers were selected for application to the HPLC columns by using a Rheodyne 5701 tandem enrichment valve driven by a Rheodyne 5704 pneumatic actuator and a Valco Digital Valve Interface (Chromtech, Apple Valley, MN).

Catecholamines were detected as they eluted from the HPLC system by using an LC-4B amperometric detector from Bioanalytical Systems (West Lafayette, IN). A glassy carbon working electrode was used at a potential of +0.65 V versus a Ag/AgCl reference electrode. Data from the HPLC/FIA system were collected using an LDC/Milton Roy Chromlink interface and LCAdvantage software. The HPLC columns were packed with an Alltech column slurry packer.

The FIA system for creatinine measurements was similar to that described previously [7]. This system consisted of a Scientific Industries Model 403 peristaltic pump (Bohemia, NY) for

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delivery of the picric acid and sodium hydroxide reagents and a Lambda-Max 481 spectrophotometer (Waters, Milford, MA) for detection of the resulting creatinine-picrate complex. The reactor used in the FIA system was a type II serpentine reaction chamber made from 1.5 m × 0.5 mm I.D. PTFE tubing [17].

2.3 Methods

Diol-bonded Nucleosil 100–7 silica was prepared according to a previous procedure [18]. 3-Aminophenylboronic acid was coupled to this support by the Schiff base method [19]. The silica used in this synthesis was sonicated under vacuum for 15 min at the beginning of the activation and immobilization steps [20]. The reaction mixture was shaken for approximately two days at room temperature during the immobilization step.

After its preparation, the resulting phenylboronic acid support (i.e., PBA silica) was washed with several portions of water, methanol and ethyl ether [19] and allowed to dry under aspirator vacuum. The PBA silica was then acetylated according to a literature method [6] by adding acetic anhydride to a suspension of the support in pyridine. This mixture was sonicated under aspirator vacuum for 15 min and shaken for 6 h at 20°C. The resulting product was washed on a fritted glass filter with several portions of water and methanol. This support was dried under vacuum overnight at room temperature. The amount of phenylboronic acid that was present on the surface of the final PBA silica was determined by carbon/nitrogen combustion analysis (Desert Analytics, Tucson, AZ).

The acetylated phenylboronic acid silica was downward slurry-packed at 4000 psi (28 MPa) into a 2.5 cm × 2.1 mm I.D. stainless steel column of a previously-published design [21]. The C_{18} Nucleosil Si-100–3 silica was similarly packed into a 10 cm × 2.1 mm I.D. stainless steel column. The mobile phase used for application of samples to the phenylboronic acid column was 0.10 mol/l phosphate buffer (pH 7.5). The mobile phase used for eluting compounds from this column and applying them onto the C_{18} reversed-phase column was a 1:10 (v/v) mixture of HPLC-grade methanol and 0.10 mol/l phosphate buffer (pH 2.5) containing 1 mmol/l Na_2EDTA and 300 mg/l sodium octylsulfate. The pH 7.5 and pH 2.5 mobile phases were applied to the HPLC/FIA system at flow-rates of 0.5 and 1.0–2.0 ml/min, respectively. Both of these mobile phases were sonicated under vacuum and filtered through a 0.45 μm nylon filter prior to use. All work on the HPLC system was performed at room temperature. The temperature of the FIA reaction coil was held at 30°C by placing this coil in a constant temperature water bath.

The following sequence of events was used for sample analysis on the final HPLC/FIA system when using a flow-rate of 1.0 ml/min on the reversed-phase column: 0.0 min, sample was injected onto the phenylboronic acid column in the presence of the pH 7.5 application buffer, with non-retained components being allowed to wash through to the FIA system; 1.3 min, the switching valve was activated and the pH 2.5 elution solvent was applied to the phenylboronic acid column to elute off any retained solutes and apply them onto the C_{18} reversed-phase column; 8.7 min, the phenylboronic acid column was switched back to the pH 7.5 application buffer and allowed to regenerate; 10 min, the analysis sequence was completed and the next sample was injected.

Experiments examining the retention of catecholamines on the PBA column when using various mobile phases were performed by making triplicate injections of standards containing 20 μg/l of norepinephrine, epinephrine or dopamine (note: this sample concentration was chosen for these particular studies to ensure that linear elution conditions were present, thus allowing accurate measurements to made of the retention factor). The binding and recovery studies conducted with catecholamines on the PBA column as a function of sample pH, mobile phase ionic strength and PBA column wash time were performed by injecting aliquots of a

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spiked urine sample that contained added catecholamine levels of 75 μg/l norepinephrine, 20 μg/l epinephrine and 220 μg/l dopamine (i.e., concentrations representative of those found in a typical sample). The solutes retained on the PBA column during these studies were later applied to the reversed-phase analytical column for separation and quantitation by ion-pair HPLC. The mobile phase conditions used with the reversed-phase column during these experiments were the same as described earlier for the final HPLC/FIA system.

The FIA system used in this work was a modified version of a previous device developed for urinary albumin measurements [7]. In this approach, the pH 7.5 application mobile phase and sample components eluting non-retained from the phenylboronic acid column were first passed through the reference cell of the FIA detector. This gave a negative peak due to the background absorbance of creatinine and other sample solutes at 521 nm. After exiting this reference cell, the sample and application buffer entered a mixing tee, where they were combined with separate solutions containing 1.5 mol/l sodium hydroxide or 0.06 mol/l picric acid in water. These latter two solutions were each delivered by a peristaltic pump at a flow-rate of approximately 0.6 ml/min. The mixture that was formed between these reagents and the column eluent was passed through the reaction coil of the FIA system and back into the sample cell of the detector. The difference in the responses measured for the sample before and after its reaction with the FIA reagents was then found to obtain the signal due to only the picrate-creatinine complex. All picric acid solutions used in this work were prepared from a suspension containing roughly 35% (w/w) water, as obtained from the supplier (note: care must be taken in the handling and disposal of this reagent because dry picric acid can be explosive upon impact).

The urine samples analyzed in this study were collected from normal college age volunteers who were participating in a study conducted by the Psychology Department at the University of Nebraska (Lincoln, NE). Immediately after collection, a 20 ml aliquot of each urine specimen was combined with 100 μl of a GSH/Na_2EDTA preservative solution and stored at −20 °C. The GSH/Na_2EDTA preservative solution was prepared fresh daily by dissolving 2.5 g Na_2EDTA and 0.5 g GSH in 50 ml water. Prior to analysis, the urine samples were thawed, diluted 1:1 with 0.1 mol/l phosphate buffer (pH 6.0), and combined with another 100 μl of the GSH/Na_2EDTA solution. The diluted samples were then filtered through disposable 0.45 μm nylon filters and placed into HPLC autosampler vials (note: as is true for any biological or clinical sample, appropriate precautions should be observed in the handling and disposal of such specimens).

Catecholamine standards for the HPLC system were prepared from a stock solution containing approximately 38 mg norepinephrine hydrochloride, 10 mg epinephrine and 110 mg dopamine hydrochloride combined with 10 ml of 0.1 mol/l perchloric acid and brought to a final volume of 100 ml with water. This stock solution was divided into 1.0 ml aliquots and stored at −20° C until use. Working solutions were prepared fresh daily by adding 100 μl of the stock standard to 5.0 ml of 0.1 mol/l phosphate buffer (pH 6.0) containing 0.5% (v/v) of the GSH/Na_2EDTA preservative solution. Standards were made by adding 5 to 100 μl aliquots of the working solution to 5 ml of 0.1 mol/l phosphate buffer (pH 6.0) containing 0.5% (v/v) of the GSH/Na_2EDTA preservative solution. A 10 g/l creatinine stock solution was prepared in water and was stored at 4°C between each use. Standards containing 0 to 2 g/l creatinine were prepared by diluting aliquots of the creatinine stock solution with 0.1 mol/l phosphate buffer (pH 6.0).

3. RESULTS AND DISCUSSION

3.1 Optimization of Phenylboronic Acid Column

The amount of ligand on the PBA support was determined by combustion analysis. The resulting coverage was found to be 260 (±20) μmol phenylboronic acid per gram of silica . If

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it is assumed that all of this ligand was available for binding, a 2.5 cm × 2.1 mm I.D. column containing this support should be able to bind approximately 2 mg of catecholamines. Less than 300 μg of total catecholamines are excreted per day into the urine of normal individuals [8]. For a daily urine output of approximately 1 l, this would correspond to a sample that has <6 ng of total catecholamines per 20 μl injection. This amount is several orders of magnitude smaller than the binding capacity that was estimated for the PBA column, thus indicating that this column had more than sufficient capacity for the extraction of catecholamines from such samples.

Since the interaction of phenylboronic acid with catecholamines is pH dependent [22], the effect of pH on the binding strength of the PBA column was next considered. This was done by measuring the retention of norepinephrine, epinephrine and dopamine on the PBA column in the presence of phosphate solutions that covered a range of pH values. The results are shown in Figure 3. Each of the catecholamines showed an increase in retention (as described by the retention factor, k'), as the pH was raised. This trend was expected because a basic pH promotes the hydroxylation of phenylboronic acid, which in turn results in a species that can undergo covalent interactions with diol-containing compounds such as catecholamines [16,22].

Based on the data in Figure 3, a pH of 7.5 was chosen for sample application onto the PBA column. This pH not only provided reasonably strong retention for all 3 catecholamines, but it also minimized any damage to the silica support that might occur when working under more basic conditions [23]. The retention factors at pH 7.5 for norepinephrine, dopamine and epinephrine were all in the range of 13.6 to 17.1. At a flow-rate of 0.5 ml/min, this retention corresponded to mean elution times of 2.0 to 2.5 min for these compounds from the PBA column. The resolution between norepinephrine and epinephrine under these conditions was 3.23 and the resolution between epinephrine and dopamine was 7.18. The average plate height (as determined from the peaks for epinephrine and dopamine) for this overall separation was 0.022 mm.

Besides indicating what application conditions should be used with the PBA column, Figure 3 also indicates what elution conditions might be employed with this column to later elute any retained catecholamines. In this case, a low pH elution buffer was chosen because these conditions produced low retention for all of the catecholamines. At pH 2.5, each of these examined catecholamines had with a mean retention time of <0.25 min (i.e., k' values of 0.62 to 0.77). These conditions resulted in the rapid release of such compounds from the PBA column. An elution pH of 2.5 was also convenient for this study in that it was fully compatible with mobile phases that have been previously used for the isocratic separation of norepinephrine, epinephrine and dopamine on reversed-phase columns [24]. This became an important feature later when the PBA column was used in combination with a C_{18} analytical column for ion-pair chromatography.

The time required for sample application onto the PBA column was another item considered in the optimization of this system. This item was of concern because enough time has to be allowed for the removal of non-specifically retained compounds from the sample; however, the application time also had to be short enough to avoid a decrease in recovery and the loss of any retained catecholamines due to their gradual elution from the PBA column under the application conditions. The maximum time that could be used for sample application was first estimated by examining the elution profiles for the catecholamines as they exited the PBA column at pH 7.5. At a flow-rate of 0.5 ml/min, no significant amount of these compounds (i.e., <2.5%) appeared up until a wash time of 1.1 min. This was confirmed by later studies in which catecholamine standards were injected onto the final HPLC system. In these experiments, a wash time of 1.3 min at 0.5 ml/min was found to give ≥90% recovery for all of the catecholamines. Similar work with spiked urine samples indicated that these conditions

were also effective in removing most non-retained sample components from the PBA column. As a result, these wash and application conditions were used in all later studies.

Since the pH of urine can vary widely from one sample to the next [8], it was necessary to consider the effect of sample pH on the binding and recovery of catecholamines by the PBA column. This was examined by making 1:1 mixtures of a spiked urine sample with six different 0.1 mol/l phosphate solutions that ranged in pH from 2.0 to 8.0. The pH of each resulting solution was then measured and a portion was injected onto the PBA column using 0.1 mol/l phosphate buffer (pH 7.5) as the application mobile phase. For norepinephrine and dopamine, the peak size obtained on the C_{18} column decreased by 15 and 11%, respectively, in going from a sample pH of 7.3 to 3.0. Epinephrine gave different behavior, with a 38% increase in peak size being observed over the same pH range. In order to obtain good reproducibility and recovery for each of these compounds, all later samples were adjusted to an intermediate pH prior to injection (i.e., approximately pH 5.5−6.0). This was accomplished by mixing each sample 1:1 (v/v) with 0.10 mol/l phosphate buffer (pH 6.0).

Several factors may have contributed to the different types of behavior that were seen for the individual catecholamines as the sample pH was varied. The slightly smaller peaks noted for norepinephrine and dopamine at an acidic pH may reflect incomplete mixing between the sample and mobile phase prior to the passage of these analytes through the PBA column. This would have resulted in a local pH that was lower than that of the mobile phase, producing weaker retention and faster elution of these compounds, as predicted by Figure 3. The increase in peak size noted for epinephrine at low pH may be due to degradation of this compound under more basic conditions. It is known that catecholamines can undergo a pH-dependent oxidation to form their corresponding quinones. In fact, this is the same process that is commonly used in the electrochemical detection of these compounds [8]. However, if oxidation of epinephrine were to occur before the sample was injected, the result would be the formation of a product which no longer has strong interactions with the PBA column. Results obtained from a previous stability study indicate that epinephrine is oxidized more rapidly than norepinephrine at pH 7.0 [25]. The higher rate of epinephrine oxidation is, therefore, a plausible reason for why larger epinephrine peaks were obtained in this current study for samples that had relatively low pH values.

3.2 Combination of Phenylboronic Acid Column with Ion-Pair HPLC and FIA Systems

After they were released from the PBA column, the catecholamines were separated on a C_{18} reversed-phased column in the presence of an ion-pairing agent (i.e., sodium octylsulfate). A typical chromatogram that was obtained on this system is shown in the lower portion of Figure 4. In this case, the norepinephrine, epinephrine and dopamine in a typical urine sample could be separated with baseline resolution in approximately 16 min.

The upper and lower portions of Figure 4 demonstrate the role that the PBA column played in sample cleanup for this analysis. The upper portion of this figure shows the results obtained for a urine sample that was injected directly onto the reversed-phase column for separation by ion-pair chromatography. The result was a series of large peaks that made it difficult or impossible to detect the desired analytes. Several additional peaks, not shown in this chromatogram, occurred at fairly long elution times (i.e., 25 − 50 min), thus requiring a significant amount of time for washing of the reversed-phase column between sample applications. When the same sample was first passed through the PBA column (see lower portion of Fig. 5), most of these background peaks were no longer detected and all were substantially reduced in size. Under these conditions, all three catecholamines could be detected at concentrations of clinical interest, as discussed later in this report. In addition, the PBA column was successful in removing most of the late-eluting sample components, thus

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minimizing the amount of time required to wash the reversed-phase column between sample injections.

A further decrease in the number and size of non-catecholamine peaks could be obtained by decreasing the concentration of the application buffer that was used on the PBA column. This factor was studied by injecting standards and spiked urine samples onto the PBA column in the presence of pH 7.5 application buffers that had phosphate concentrations ranging from 0.001 to 0.10 mol/l. Under these conditions, there was little or no change in the size of the catecholamine peaks that eluted from the reversed-phase column. However, there was a decrease in peaks due to other sample components as the phosphate concentration of the application buffer was lowered. This is probably due to the promotion of non-polar interactions between sample components and the aromatic ring of phenylboronic acid at high buffer concentrations, as has been observed for other systems [16]. In this particular study this effect was only marginally useful because there were already relatively few peaks present even when using the 0.10 mol/l application buffer. In addition, none of the other remaining peaks that were observed had any significant overlap with peaks due to norepinephrine, epinephrine and dopamine. Thus, the practice of using a 0.10 mol/l application buffer (pH 7.5) was continued throughout the remainder of this report.

Solutes eluting in the non-retained fraction of the PBA column were washed with the pH 7.5 application buffer into an on-line FIA system for the determination of creatinine. The reaction conditions used in this FIA method were similar to those described previously for use in determining urinary albumin [7]. This part of the overall analysis system included a means for determining the absorbance of the sample both before and after addition of the alkaline picrate reagent. As shown in Figure 2, this was accomplished by first passing the non-retained fraction of each sample through the reference side of the FIA detector before reagent addition. This gave a negative peak following injection that was related to the background absorbance of the sample components (see top of Fig. 5). After these components has passed through the reference cell of the FIA detector, they were combined with the sodium hydroxide and picric acid reagents, allowed to react, and passed through the sample side of the same absorbance detector. This gave a second, positive peak that was related to the sample background plus the absorbance of the newly-formed picrate-creatinine complex. By comparing the signals measured before and after reagent addition, the signal due to only the picrate-creatinine complex was obtained [7].

One difference between this FIA system and that described previously for urinary albumin was that a slightly higher pH for the application buffer was required by the affinity column used in this current study (i.e., pH 7.5 vs pH 7.0) [7]. This difference was important to consider because formation of the picrate-creatinine complex in the FIA system is a pH-dependent process [8]. In this study, the higher pH of the application buffer was offset by using a lower concentration of sodium hydroxide for the FIA reaction. The optimum response of the FIA system, as determined by making replicate injections of a 2 g/l creatinine standard, was found to occur when a sodium hydroxide concentration of 1.5 mol/l was used. As a result, this was the concentration of sodium hydroxide used in all later studies with the system shown in Figure 2.

Before this FIA system could be combined with the PBA column, it was necessary to test for retention of creatinine by the PBA support. This was first examined by injecting 2 g/l creatinine standards directly onto the PBA column while monitoring the absorbance of the column eluent. The peaks that were detected for creatinine appeared at or near the column void time. This indicated that creatinine was passing almost directly through the PBA column. Creatinine retention was further examined by making calibration curves for the FIA system based on creatinine standards that were injected both with and without the PBA column being present.

No noticeable differences were observed in the resulting curves, indicating that the PBA column did not interfere with detection of creatinine by the FIA system.

3.3 System Performance and Evaluation

Figure 5 shows some typical results that were obtained for multiple injections of a urine sample onto the final HPLC/FIA system. The top tracing shows the response due to creatinine on the FIA system and the bottom result shows the response obtained for the catecholamines on the HPLC component of the system. These plots indicate that good reproducibility and a reasonably high sample throughput were obtained by this method. When using a flow-rate of 1 ml/min for the reversed-phase C_{18} column (see Fig. 4), the total analysis time was approximately 16 min per injection. By working at a flow-rate of 2 ml/min on the same column (see Fig. 5), a potential throughput of one sample every 10 min could be obtained.

Table 1 summarizes the properties that were obtained for calibration curves of this system for mixed standards containing norepinephrine, epinephrine and dopamine. In this table, the lower detection limit was defined as the concentration of analyte that gave a response with a signal-to-noise ratio = 3 vs the injection of a buffer blank; this detection limit (LOD) was 1.0−4.3 μg/l, with epinephrine having the lowest value and dopamine giving the highest value. The upper limit of the linear range was determined by using the maximum analyte concentration that gave a response within ±5% of that predicted by the best-fit line. The result was an overall linear range that extended over a 450 to 930-fold change in concentration for the catecholamines that were examined in this study. The upper limit of the dynamic range was defined as being that level of analyte at the upper end of the calibration curve which gave a minimum change in response of 5% from the signal measured for a large excess of analyte. This dynamic range included the linear range of the method and covered a response that extended over a 600 to 1400-fold change in concentration for the catecholamines.

The LODs shown in Table 1 are comparable to those that have been reported in previous work using HPLC with fluorescence detection or electrochemical detection (see review in Ref. [26]). Lower detection limits of 0.02−0.12 μg/l have been reported for norepinephrine, epinephrine, and dopamine when measured by a radioenzymatic method, but this approach involves the use of a tritiated reagent [8]. The combined use of HPLC or capillary electrophoresis with chemiluminescence detection has also been described for the analysis of catecholamines, in which the presence of catecholamines can either inhibit [27] or enhance signal intensity [28,29]. These latter methods have resulted in detection limits that are comparable to or slightly lower than those obtained in this previous report (i.e., 0.01−8 μg/l), but these methods do require a post-column/capillary system for the initiation and detection of chemiluminescence [27-29].

For a daily urine output of one liter, the normal urine concentrations in adults that would be expected for the catecholamines are 15−80 μg/l norephinephrine, 0−20 μg/l epinephrine and 65−400 μg/l dopamine [30]. The data in Table 1 indicates that these concentrations are all well within the linear range of the described method. The same data also indicate that the limits of detection and linear range of this technique are sufficient for the analysis of urine samples that might contain levels of catecholamines that are much smaller or greater than those found in healthy adults. For example, one recent study using 24 h urine samples obtained from patients with phaeochromocytoma found that norepinephrine and epinephrine increased to more than double their upper reference limits in 80% of these patients [13], a result which would fall well within the linear range for the method developed in this current study. The results in Table 1 indicate that concentrations that are even up to 10 to 22-fold higher than the upper limit of the normal range would give a linear response in this method.

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A typical calibration curve prepared for creatinine on this system gave a linear response over the range of 0−2.5 g/l, with a correlation coefficient of 0.9985 over 7 data points. The lower limit of detection at S/N = 3 was estimated to be 5.0 mg/l and the dynamic range extended up to at least 10 g/l. This range also includes typical values that are expected for healthy individuals or those with a variety of disease states [30].

The accuracy of the HPAC/FIA method was evaluated by using this technique to analyze a urine control sample. In this case, the total amount of free norepinephrine, epinephrine and dopamine in the sample was determined to be 236 (± 14) μg/l, where the value in parentheses represents a range of ± 2 S.D. The reference value for the total free catecholamines in this sample was 175 (± 50) μg/l. These results differed by 35% but were within two standard deviations of each other's values. For creatinine, the reference value given for the same control sample was 1.17 (± 0.19) g/l, while the concentration measured by the HPLC/FIA system was 1.04 (± 0.03) g/l. These results differed by 4.0% and also fell within a range of two standard deviations of each another.

The within-day precision of the HPLC/FIA method was evaluated by making 20 sequential injections of a urine control sample. This urine contained mean free catecholamine levels of 162 μg/l norepinephrine, 14.6 μg/l epinephrine and 60 μg/l dopamine and a creatinine concentration of 1.0 g/l. The relative variations seen in results for this sample were ± 0.9% for norepinephrine, ± 2.7% for epinephrine, ± 2.0% for dopamine and ± 2.7% for creatinine.

Over the course of hundreds of sample injections onto the HPLC/FIA system, relatively few sample peaks were noted that overlapped with those of norepinephrine, epinephrine and dopamine. There was one peak of unknown identity (possibly representing a catecholamine metabolite) that did appear near the retention time for epinephrine, but these peaks were still sufficiently resolved to allow the measurement of epinephrine at levels of clinical interest. In previous work, the specificity of the FIA method for creatinine was examined in presence of a number of potential interferences. This was studied by injecting urine samples spiked with increasing amounts of several compounds known to interfere with the manual Jaffe method [8]. It was found that there were no significant interferences from glucose, ascorbic acid, acetone, albumin, acetoacetate, oxaloacetate or α-ketoglutarate at typical clinical levels. An added advantage of the method developed in this report is that dopamine, which can create significant interferences in the Jaffe method [8], is extracted and removed by the PBA column before it can enter the FIA part of the system.

The ability of this method to simultaneously monitor both creatinine and other analytes makes this HPLC/FIA system an attractive method for use in monitoring urinary compounds. With this approach it was possible to provide relatively fast results for small volumes of random urine samples that were collected as part of a psychological study. However, the same method could be utilized with 12 or 24 h urine specimens. By using different types of affinity extraction and analytical columns, this same general approach of combining HPLC and FIA could be adapted for use in the measurement of other solutes in urine, as has been already demonstrated in the previous use of a similar FIA method with an immunoaffinity column for the determination of urinary albumin [7].

Acknowledgements

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List of Abbreviations

FIA, flow injection analysis; glutathione, GSH; HPAC, high performance affinity chromatography; Na_2EDTA, disodium ethylenediaminetetracetic acid; PBA, phenylboronic acid; RPLC, reversed-phase liquid chromatography.

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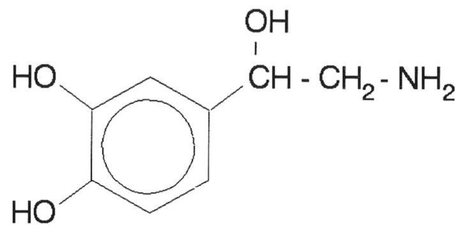

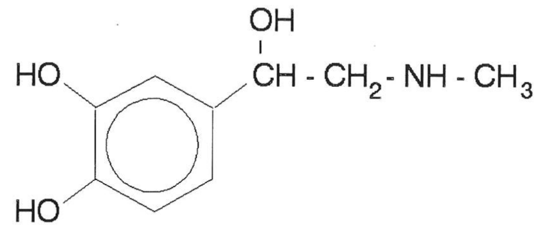

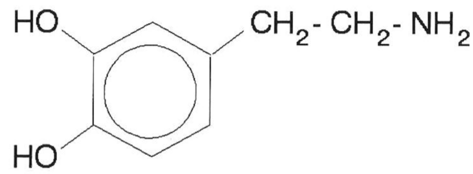

Figure 1.
Structures of epinephrine, norepinephrine and dopamine.

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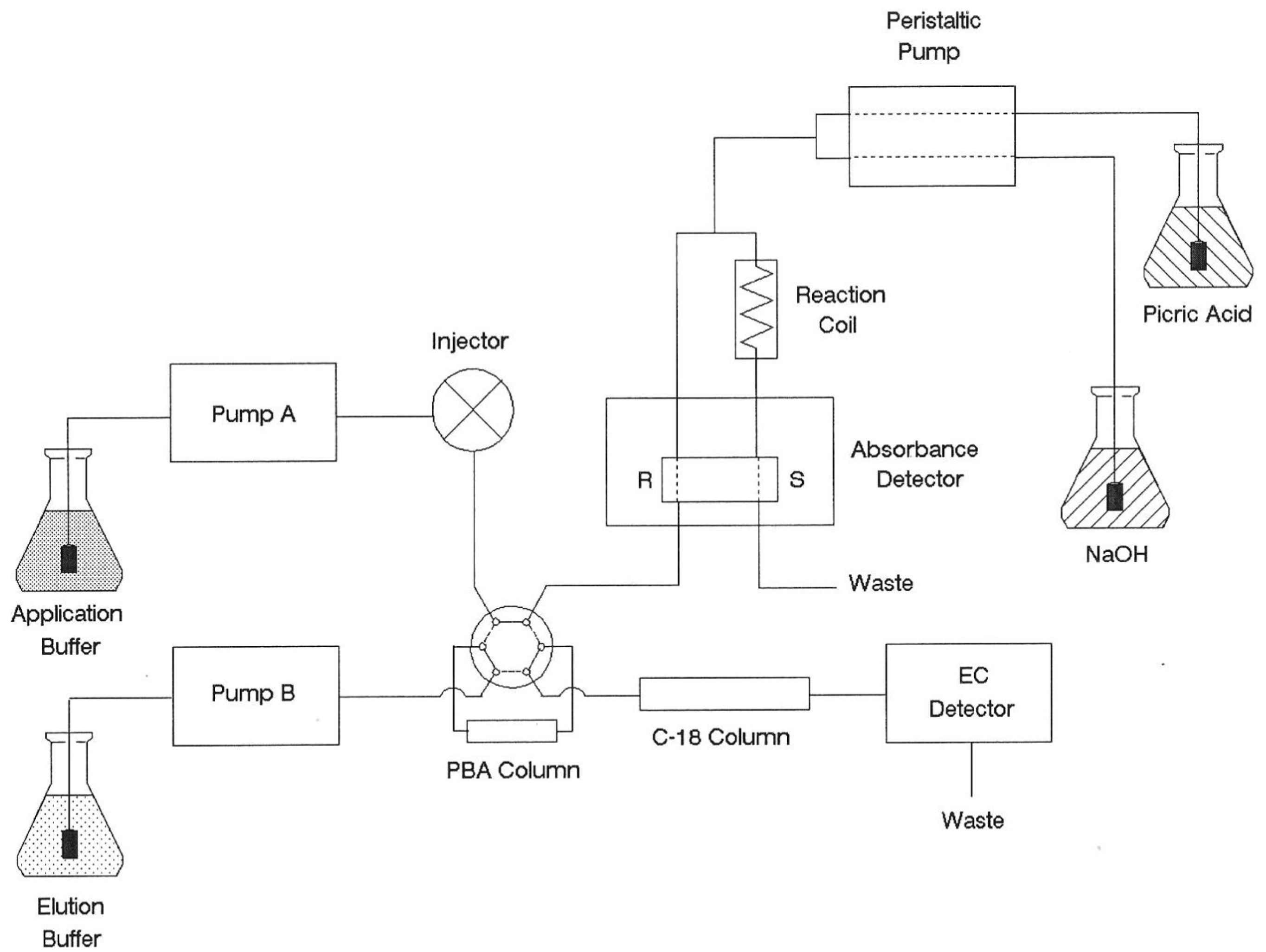

Figure 2.
Schematic of an HPLC/FIA system for the measurement of free catecholamines in urine. The positions of reference and sample cells in the FIA detector are designated by “R” and “S”, respectively. The term “PBA” refers to the phenylboronic acid support.

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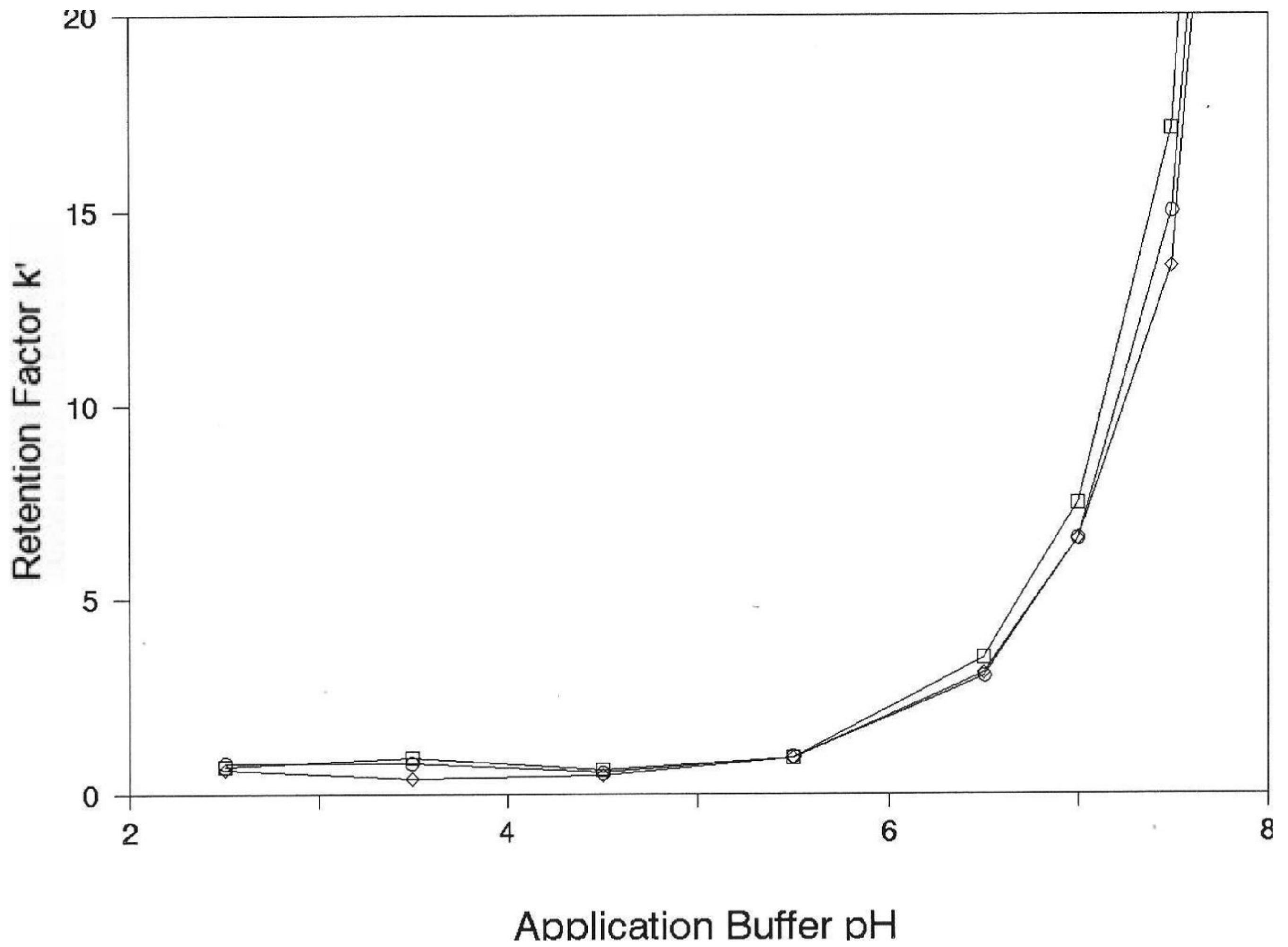

Figure 3.

Retention factors (k') for dopamine (□), norepinephrine (○) and epinephrine (◇) on the PBA column in the presence of 0.10 mol/l phosphate buffer at various pH values. Other experimental conditions are given in the text.

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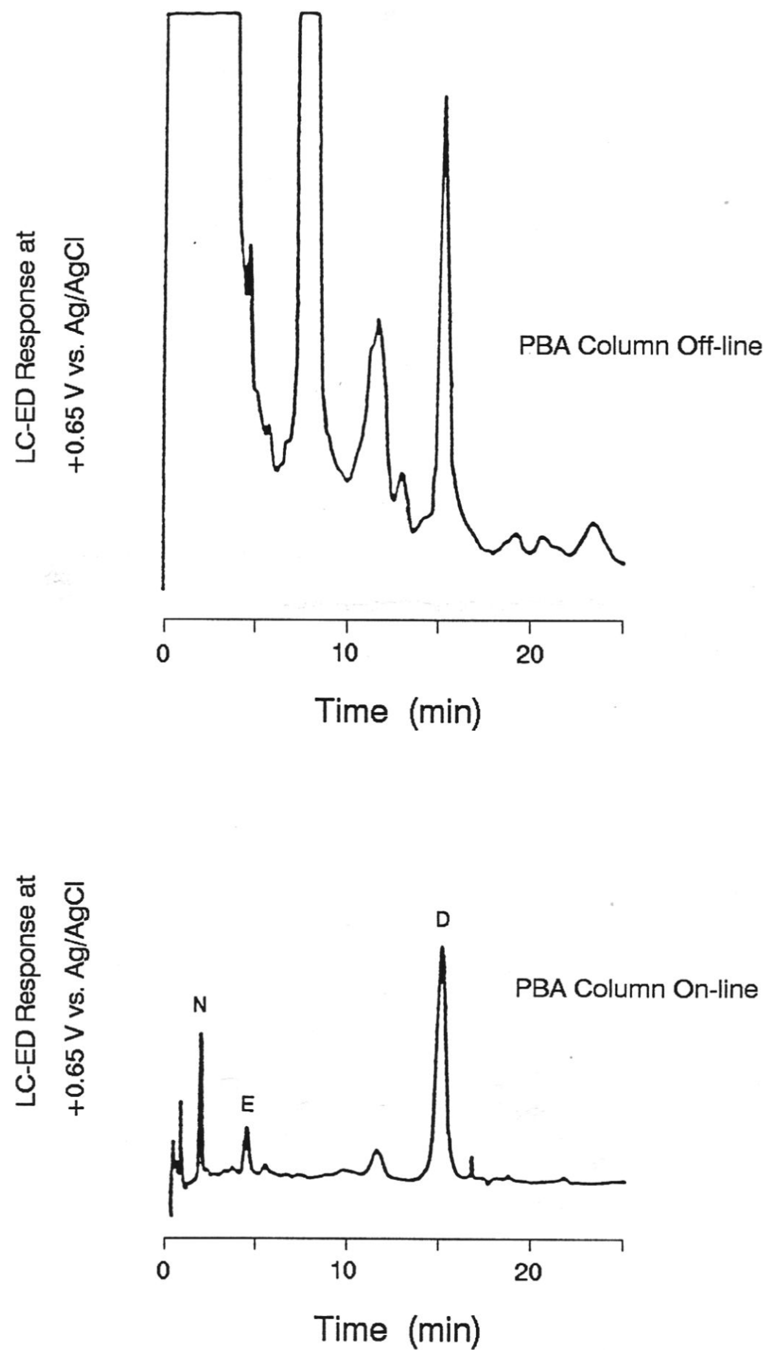

Figure 4.

Typical chromatograms obtained for an injection of urine onto the ion-pair reversed-phase HPLC system (a) before and (b) after passing through the PBA column. The mobile phase flow-rate through the reversed-phase column was 1.0 ml/min. All other conditions were the same as described in the text for the final HPLC/FIA system.

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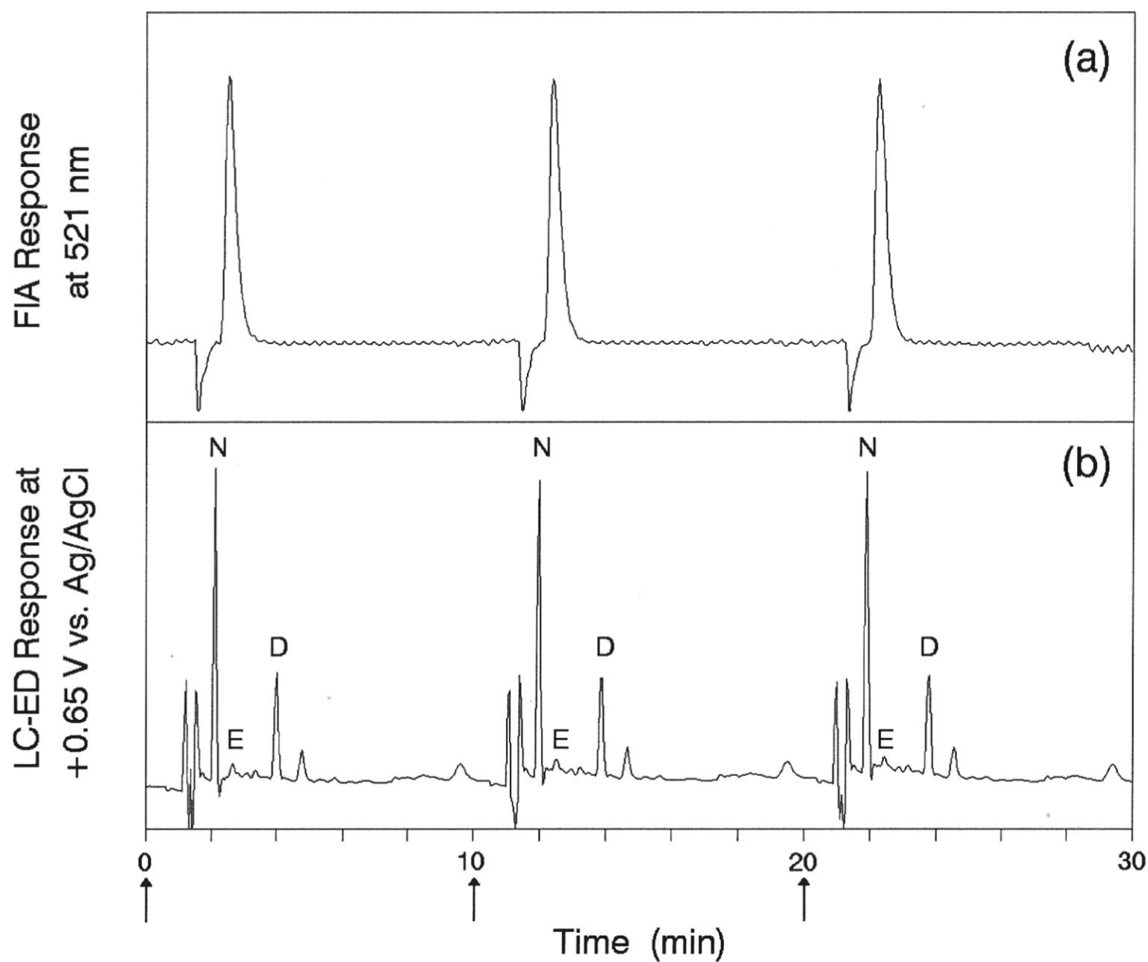

Figure 5.
Typical results obtained for injections of urine on the HPLC/FIA system. The top portion shows the response of the FIA system at 510 nm. The bottom portion shows the response obtained at the electrochemical detector following compound elution from the C_{18} reversed-phase column. The mobile phase flow-rate through the reversed-phase column was 2.0 ml/min.

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Table 1

Typical properties for the calibration curves obtained for catecholamines on the tandem affinity/ion-pair HPLC and FIA System

	Norepinephrine	Epinephrine	Dopamine
Overall Curve Parameters:[a]			
Detection Limit (μg/l)	1.8	1.0	4.3
Upper Limit of Linear Range (μg/l)	1150	450	4000
Upper Limit of Dynamic Range (μg/l)	2000	600	6000
Linear Best-Fit Parameters:[b]			
Slope (nA-L/μg)	0.543 (± 0.001)	0.425 (± 0.002)	0.248 (± 0.001)
Intercept (nA)	−0.1 (± 0.9)	−0.1 (± 0.5)	−0.2 (± 1.0)
Correlation Coefficient	0.9999	0.9999	0.9999

[a]All values were obtained at a flow-rate of 1.0 ml/min on the reversed-phase column. The definitions used for the individual parameters are provided in the text.

[b]The best-fit parameters shown were measured over 9−11 standards spread throughout the stated linear range. The numbers in parentheses represent a range of ± 1 S.D.