evaluation of a new method for the analysis of free catecholamines in plasma using automated sample trace enrichment with dialysis and HPLC

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Background: Analysis of urinary free catecholamines was automated recently, but analysis of plasma samples posed special difficulties. The present study was undertaken to evaluate a new method for the automated analysis of plasma catecholamines.

Methods: The procedure is based on an improved sample handling system that includes dialysis and sample clean-up on a strong cation trace-enrichment cartridge. The catecholamines norepinephrine, epinephrine, and dopamine are then separated by reversed-phase ion-pair chromatography and quantified by electrochemical detection.

Results: Use of a 740-μL sample is required to give the catecholamine detection limit of 0.05 nmol/L and analytical imprecision (CV) between 1.1% and 9.3%. The assay can be run unattended, although >12 h of analysis time is not recommended without cooling of the autosampler rack. Comparison (n = 68) of the automated cation-exchange clean-up with the well-established manual alumina procedure gave excellent agreement (mean, 3.78 ± 2.76 and 3.8 ± 2.89 nmol/L for norepinephrine and 0.99 ± 1.72 and 1.08 ± 1.78 nmol/L for epinephrine). Hemodialysis had no clear effect on plasma norepinephrine. Epinephrine concentrations were similar (0.05 < P < 0.1) in chronic renal failure patients (0.24 ± 0.3 nmol/L; n = 15) and healthy controls (0.5 ± 0.24 nmol/L; n = 31). Dopamine was not quantified, being usually <0.2 nmol/L.

Conclusion: The availability of such a fully automated procedure should encourage the more widespread use of plasma catecholamine estimation, e.g., after dialysis, exercise, or trauma/surgery and in the investigation of catecholamine-secreting tumors, particularly in the anuric patient.

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The routine analysis of plasma free norepinephrine, epinephrine, and dopamine is fraught with analytical problems, primarily associated with low plasma concentrations and the relatively labor-intensive manual extraction procedure required to remove interfering compounds (1). The automation of such techniques using automated sequential trace enrichment of dialysates (ASTED)3 has been applied successfully to the assay of urinary free catecholamines (2). The application to plasma, however, presented specific problems; in particular, the original ASTED system could not achieve regeneration and conditioning of both the dialyzer and the trace-enrichment cartridge (TEC). This has now been successfully resolved with the development of the second generation ASTED.XL™, which utilizes two rheodyne valves and two dilutors. The technique involves the dialysis of the plasma sample to remove protein and other macromolecules. The catecholamines are then trace-enriched onto a strong cation-exchange resin (3) and eluted onto the analytical column. Separation is achieved by reversed-phase ion-pair chromatography with coulometric detection of the catecholamines (4), whereas the TEC and dialyzer are regenerated for subsequent injections. Procedures reported previously for automated plasma catecholamine analysis have included a manual clean-up step or simple deproteinization (5) and then solid-phase extraction (6) followed by automated HPLC with electrochemical or fluorescence detection (7).

We here report the analytical evaluation of the fully automated analytical ASTED procedure for measuring
plasma catecholamines. The analysis of plasma norepinephrine (NE) and epinephrine (E) was compared in healthy volunteers, patients being investigated for catecholamine-secreting tumors, patients with chronic renal failure, and in particular, assessment of the effect of regular hemodialysis in patients with end-stage renal failure.

**Materials and Methods**

Blood samples (10 mL) were collected into tubes containing lithium heparin as the anticoagulant (Sarstedt) and transported to the laboratory on ice within 30 min. Sampling lithium heparin as the anticoagulant (Sarstedt) and blood samples (10 mL) were collected into tubes containing lithium heparin as the anticoagulant (Sarstedt) and dialyzed into 2 mL of 5 mmol/L ammonium hydrogen orthophosphate (BDH) containing 101 mg of heptane sulphonatic acid (Sigma Chemical Co.) and 73 mg of EDTA (Sigma) adjusted to pH 3.5. A 15.0 x 0.46 cm Ultradeosphere 5 µm ODS 2 column (HPLC Technology) was used at a flow rate of 1.5 mL/min. The dialysis recipient solvent was 5 mmol/L diammonium hydrogen orthophosphate adjusted to pH 8.3. Dihydroxybenzylamine was used as the internal standard (IS) at a concentration of 60 nmol/L, 50 µL of which was added to 750 µL of sample.

The effect of the serum matrix on dialysis was assessed by comparison of peak areas after injection onto the complete system of an aqueous calibrator, a serum blank, and the same serum to which 10, 5, 20, or 5 nmol/L NE, E, dopamine (D), and IS had been added. The mean ± 1 SD recoveries (n = 5) were 83% ± 2%, 75% ± 2.5%, 95% ± 3.8%, and 91% ± 3%, respectively, which indicated a small loss because of the protein matrix effect. The relative recovery through the TEC without dialysis was compared with loop injection only using 100 µL of an aqueous solution containing 10 nmol/L NE, 5 nmol/L E, 20 nmol/L D, and 5 nmol/L IS. The recoveries (mean ± SD; n = 4) were 89% ± 1.5%, 77% ± 1.5%, 91% ± 2%, and 94% ± 2.1%, respectively, indicating some loss on the TEC, in particular for E because of its reduced retention on the cation exchanger (2). The overall recovery or efficiency of the dialysis was assessed by comparison of the peak areas after injection of an aqueous calibrator (through the loop only) and a serum blank supplemented with 10 nmol/L NE, 5 nmol/L E, 20 nmol/L D, and 5 nmol/L IS taken through the complete system. The recoveries were 38% ± 2% for NE, 35% ± 1.5% for E, 19% ± 0.8% for D, and 34% ± 1.7% for IS (mean ± 1 SD; n = 4), indicating a relatively efficient dialysis.

The system was calibrated using standard additions of NE (Sigma) at 0, 5, 10, 15, and 20 nmol/L and E (Sigma) at 0, 4, 8, and 10 nmol/L to drug-free serum (Bio-Rad). The individual catecholamine responses were corrected to the IS response, and this ratio was related to known concentrations of each analyte. The regression analysis of n = 6 observations for NE and E was: y = 11.1 x 10^x + 10, r = 0.998; and y = 12.5 x 10^5 x + 8, r = 0.988, respectively, where y is the electronic signal in computed integration units (Gilson Unipoint Software). In routine use, the calibration was confirmed with 0 and 10 nmol/L NE and 0 and 5 nmol/L E. Calibrators were run every tenth sample, and controls were run every fifth sample. If the signal response deviated >15%, then recalibration would be carried out to ensure that the controls were always within 1 SD of the expected mean value. The analytical performance (Table 1), was monitored with commercially available Endocrine Controls 1 and 2 (Bio-Rad A and B). All solutions were prepared using doubly deionized water (Elga Products Ltd).

The alumina extraction (9) was used as the comparative reference procedure with 69 plasma samples obtained from the various groups outlined above. The catecholamines from a 1.0-mL plasma sample were desorbed
from alumina with 400 μL of 0.1 mol/L phosphoric acid (Sigma), and 370 μL was injected for separation by HPLC as outlined for the ASTED procedure. The system was controlled and analytical integrations were carried out by Gilson UniPoint Software with a Gilson 506C interface module (Anachem).

All data were processed using Microsoft Excel for Office 95, and data were compared with standard linear regression analysis and differences between groups with parametric Tukey and nonparametric Kruskal–Wallis statistical analysis, using the ARCUS software as supplied by the University of Liverpool, Liverpool, UK.

### Results

Typical chromatograms obtained for automated plasma catecholamine analysis are shown in Fig. 1. The analytical performance given in Table 1 indicates good precision. The detection limit, under the conditions outlined and determined as 3 SD above the mean background signal noise, was 0.05 nmol/L for both E and NE with a 740-μL plasma volume injected. The commonly prescribed beta blockers, angiotensin-converting enzyme (ACE) inhibitors, l-dihydroxyphenylalanine, and metabolites from caffeine or acetaminophen did not interfere in the assay.

Stability of the catecholamines on the system (i.e., on the autosampler rack at 20–22 °C) was assessed using various control solutions and indicated a loss of activity of both NE and E of 1.0%/h in plasma and 3.0%/h in an aqueous solution. Plasma samples stored ≥72 h at 4 °C decayed at 0.2%/h, over which time a 15% loss was considered significant. The routine practice, therefore, is to calibrate with serum-based calibrators and to perform the analysis in batches such that samples remain on the autosampler for periods no longer than 10–12 h. Analytical comparison with the reference alumina extraction procedure (Fig. 2) gave excellent agreement for NE and E over a wide range of concentrations, showing no significant difference between the two analytical procedures.

Plasma catecholamines varied little among the patient groups studied (Fig. 3). Plasma NE was significantly increased in the predialysis and hospital patients compared with the healthy controls, \( P < 0.05 \), but pre- and postdialysis concentrations were not significantly different. E was relatively higher in the healthy volunteers. In the chronic renal failure patients, plasma NE was 2.0–5.8 nmol/L and E was 0.1–0.7 nmol/L, both independent of creatinine clearance in the range 5–120 mL/min. In patients investigated for a catecholamine-secreting tumor, plasma NE and E concentrations similar to healthy controls would suggest exclusion of such a diagnosis. One patient (data not in Fig. 3) with increased NE (11.8 and 9.6 nmol/L), a strong clinical suspicion, and a negative clonidine suppression test was subsequently investigated with selective venous sampling, which identified a catecholamine-secreting tumor in the inferior vena fossa.

In the volunteers studied after exercise, the plasma NE rose from (mean \( ± 1 \text{ SD} \) 4.5 \( ± 2.5 \) nmol/L (range, 1.5–6.4...
nmol/L) to 15.6 ± 5.6 nmol/L (range, 8.0–35.2 nmol/L), and E rose from 0.5 ± 0.3 nmol/L (range, 0.2–1.0 nmol/L) to 4.5 ± 1.5 nmol/L (range, 1.6–8.6 nmol/L).

**Discussion**

We have shown that the fully automated quantitative analysis of the plasma free catecholamines NE, E, and D can be achieved without any sample pretreatment, using the newly developed ASTED.XL system. D was not usually measured under these conditions (being near the analytical detection limit); however, it can be seen at higher concentrations, and therefore more reliably quantified, after treatment with sulphatase (7) or when patients are on l-dihydroxyphenylalanine (10). This methodological advance has been made possible because of the incorporation of improved software to control two rheodyne valves that allow access to the two dilutors to regenerate the TEC and recondition the dialyzer block independently. In the ASTED configuration for urine analysis (2), dialysis was not required and only one dilutor was made available for regeneration of the TEC.

However, when the ASTED.XL was used, both dialysis and trace enrichment could be controlled easily. The conditions were then optimized on 740 μL of plasma. If measurement of concentrations below or near the detection limit is required, more sample, e.g., 1000 μL of plasma or a longer dialysis time could be used.

We have been performing the procedure routinely for several years, having originally demonstrated that the system operates satisfactorily for plasma catecholamine analysis (11). To maintain good performance, reagents must be prepared fresh weekly, using pure water as outlined. Any loss of sensitivity other than that caused by old reagents can often be traced either to poor trace enrichment or to poor dialysis, although the dialyzer should remain effective for up to 6 months. The TEC and the analytical column should be functional for a similar period or a minimum of up to 2–3000 injections. The
concentrations were generally the same in all groups and were increased in both patients on regular dialysis and those in varying stages of renal failure but not on dialysis compared with healthy controls. Although the range of results was similar, some of the end-stage renal failure patients would appear to be more stressed than others. Clearly, additional studies are required to explain the mechanism of this phenomenon and whether the stressed patients are more likely to have some form of autonomic nerve dysfunction associated with renal disease (19). E concentrations were generally the same in all groups and were not affected by dialysis, although concentrations were shown to be increased in the healthy volunteers. A particular benefit of the plasma assay for patients in end-stage renal failure is the ability to identify catecholamine-secreting tumors. This is highlighted by the recent report of an anuric patient presenting with paroxysmal hypertension suspected to be as a result of a dopamine-secreting tumor (20).

In conclusion, a procedure has been developed for the automated assay of plasma catecholamines. The assay is suitable for routine clinical applications, is relatively inexpensive to perform, and is sensitive and precise. This procedure will facilitate routine measurement of catecholamines and therefore, additional studies of changes in health and disease, in particular, renal disease and the investigation of catecholamine-secreting tumors in patients who are anuric.

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References


