Basal Plasma-Catecholamine-Level Determination Using HPLC-ED and Different Sample Cleanup Techniques

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Key Words
Basal catecholamine plasma levels
HPLC with electrochemical detection
Picogram – detection limit
Sample cleanup

Summary
Reversed phase HPLC with electrochemical detection was used for the determination of basal adrenaline, dopamine and noradrenaline levels in human plasma. These compounds demonstrated good stability during different stages of collection and long-term storage.

Using a new electrochemical detector and improving mobile phase parameters, we obtained a detection limit of 2 pg per injection. Good separation of dihydroxyphenylacetic acid was also attained, which is important in investigations with intensive care patients.

Good accuracy and precision, demonstrated in the daily quality control measurements taken over a five month period, verified the reliability of the chromatographic separation.

However, there was a decrease in the recovery of very low amounts of catecholamines, added to fresh frozen plasma that had previously been made catecholamine-free. According to the widely-accepted extraction method of Anton and Sayre, it is argued that the unknown affinity of catecholamines to acid-prepared aluminium oxide (in comparison to catecholamine – protein binding constants) explains the low accuracy in measurement at very low plasma levels. We thus compared this sample preparation method to recently published extraction procedures.

Introduction
An understanding of the role of biogenic amines in central nervous system functions and cardiovascular regulation, especially regarding the neuroendocrine stress response, has depended upon the development of highly sensitive and accurate assays for noradrenaline, adrenaline, and dopamine. This method of analysis is still valid, even though several objections have been made concerning the use of plasma values as an index of overall sympathetic nervous system activity [1].

While fluorimetric assays did not provide reasonable assay sensitivity in the sixties, ten years later radioenzymatic procedures permitted the determination of catecholamines at low picogram levels (for review see [2]).

During the last decade, most analysts have preferred high pressure liquid chromatography, despite the disadvantage of slightly lower sensitivity. Since its introduction in 1973 [3], high pressure liquid chromatography with electrochemical detection (HPLC-ED) in particular has gained popularity, undergoing many of modifications in the process [4–9]. The number of publications indicates that several issues still remain unresolved, including the question of sufficient sensitivity for basal plasma concentrations of adrenaline (A: 20–60 pg/ml) and dopamine (DA: 0–20 pg/ml). Another problem concerns the optimization or replacement of the uncritically applied extraction procedure proposed by Anton and Sayre [10], which is unspecific and, due to low recoveries, aggravates the problem of low plasma concentration. Sensitivity for basal noradrenaline levels (NA: 200–400 pg/ml) is given with most techniques.

The aim of this study was to measure basal adrenaline and dopamine plasma concentrations. Radioenzymatic procedures are sensitive, but labour-intensive and expensive, so sample throughput is limited. Therefore we chose HPLC – ED on reversed phase columns.

In this paper we present a procedure that was applied routinely to a large number of samples (more than 4000/year). Accuracy and precision were determined to provide quality control of routine measurements. In addition, several different extraction procedures were used to obtain the following results.

Materials and Methods
The method routinely used for prepurification of plasma is adsorption of catecholamines to acid washed alumina at pH 8.6, followed by desorption with acid [10]. For the

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separation and quantification of catecholamines, ion-pair chromatography with amperometric detection at a potential of 0.6 V was utilized.

The internal standard dihydroxybenzylamine-hydrobromide and the catecholamines noradrenaline-hydrochloride, adrenaline-bitartrate, dopamine-hydrochloride and homovanillic acid, hydroxyindole acetic acid and isoproteneol-hydrochloride were purchased from Sigma Deisenhofen, FRG; dihydroxyphenylacetic acid (DOPAC) and caffeic acid were obtained from Serva, Heidelberg, FRG, as well as tетraoxyctylammoniumbromide, serotonincreatinine-sulfate and sodiumoctylsulfonate from Fluka, Neu-Ulm, FRG; diphenylborinethanolamine from Aldrich, Steinheim, FRG; dibutyramine from Waters, Eschborn, FRG; Seprlyte SCX from Analytichem (Ict, Frankfurt, FRG); Trisbuffer from Sigma Deisenhofen, FRG; dihydroxyphenylacetic acid (DOPAC) and caffeic acid were obtained from Serva, Heidelberg, FRG, as well as tетraoxyctylammoniumbromide, serotonincreatinine-sulfate and sodiumoctylsulfonate from Fluka, Neu-Ulm, FRG; diphenylborinethanolamine from Aldrich, Steinheim, FRG; dibutyramine from Waters, Eschborn, FRG; Seprlyte SCX from Analytichem (Ict, Frankfurt, FRG). All other chemicals were of the purest grade available.

Alumina was stored in a desiccator until use; all amines were stored in a refrigerator, as were plasma controls, standards and internal standards, used within one week. Stock solutions of standards and internal standards were prepared by dissolving 1-3mg of the substances in a solution of 0.2Mol/l acetic acid, as well as 250mg EDTA and 500mg sodiumdisulfite per liter. They were then stored in a deep freezer at -80°C for one year. This solution was identical to the one used to elute the catecholamines from the alumina.

Chromatographic Equipment

The mobile phase is delivered by a constametric pump (Model 590), equipped with extra pulse dampeners to a U6K sample injector or a Wisp 710 B autosampler. The quality of the Resolve® column (Waters, suitable dimensions 125 x 4.6mm, 5 μm particles) exceeded 3000 plates for the given system, calculated using the 5σ method. The signal of the electrochemical detector M460 was evaluated using a integrator 730 (all equipment Waters, Eschborn, FRG) and compared to the internal standard using the peak area mode. This electrochemical detector was recently developed by Rall, München, FRG. With the reference electrode skillfully integrated in the analytical (2.5μl, equipped with a glassy carbon electrode), including an amplifier with a very low background noise, high sensitivity was achieved without the need of high time constants or filters.

The mobile phase for reversed phase separation of the catecholamines was buffered to pH 4.5 using sodiumacetate 25mMol/l and citric acid 10mMol/l. It contained sodiumoctylsulfonate (SOS) 1.5mMol/l as an ion-pairing-agent, dibutyramine 0.5mMol/l to reduce adsorption processes and EDTA-sodium. The column-to-column performance differences were compensated varying the concentration of methanol between 5 and 10 percent (Vol/Vol). In columns that showed extreme differences in retention times, it was sometimes necessary to change the SOS concentration. Overnight and during measurement of external standards, the eluent was recirculated. The eluent used for plasma analyses was pumped to waste.

Sample Preparation

Blood was drawn in commercially available lithiumheparinate-monovettes. When drawn from healthy volunteers in the laboratory, blood was centrifuged within one hour and the supernatant was stabilized using a solution containing 61g of Glutathione and 76g of EGTA per liter [11]. For blood samples derived from clinical patients, the above-mentioned monovettes were prepared with 10μl of the stabilizing solution per ml blood. Stabilized plasma was kept stable in the deep freezer at -80°C for one year. The refreezing or rethawing of samples is deleterious; in sample eluates and standards it will cause a loss in catecholamine content.

Alumina Extraction

50μl of the internal standard solution (DHBA 10μg/l) was added to 1ml plasma in a 2.5ml reaction vial, followed by 10mg alumina and 400μl Tris-buffer (2Mol/l, pH 8.5). The plasma was extracted by overhead shaking for 15min, then the alumina was settled down by centrifuging (10000g). After removing the plasma, the alumina was washed three times with 0.02Mol/l Tris-buffer. Care was taken to remove as much of the last washing as possible before eluting the amines. This was done by using 100μl of 0.2Mol/l acetic acid, as described above and by overhead shaking for 15 minutes. After centrifugation, the supernatant was transferred to an injection bottle, taking care not to transfer alumina particles. 10–50μl of this eluate was then injected into the chromatographic system.

Solvent Extraction [12, 4]

To isolate catecholamines outside of plasma, using solvent extraction and complexing with diphenylborinethanolamine (DPBEA), 1ml plasma was extracted with 1ml buffer (ammoniumchloride 2Mol/l, DPBEA 8Mol/l, EDTA; pH 8.5) and 5ml organic tetraoctylammoniumbromide solution by vortex mixing for 5 minutes. The organic layer was diluted and reextracted with 100μl of the desorbing solution described above.

Extraction Using a Cation Exchange Step and Solvent Extraction [13]

For this isolation procedure 1ml plasma was extracted with 50mg Seprlyte SCX cation exchange resin (the same as in Bond Elut SCX columns), which was activated by phosphate buffer. The resin was washed twice with water before desorbing the amines with 1ml sodiumdihydrogenphosphate buffer (1Mol/l, pH 2.9). The supernatant was treated as described above, using the organic extraction procedure.

Results

The composition of the mobile phase is a modification of the eluent Schleicher and Kees [14] used for analysis of