Journal of Chromatography, 493 (1989) 157–163
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO. 4786

Note

Highly sensitive determination of catecholamine and serotonin concentrations in plasma by liquid chromatography–electrochemistry

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(First received September 9th, 1988; revised manuscript received March 8th, 1989)

High perfôrmance liquid chromatography with electrochemical detection (HPLC–ED) has been used for sensitive and specific detection of catechol- and indoleamines in human or animal plasma [1–5], brain homogenates [6–9], and other biological fluids [2–5,10]. Reversed-phase C_{18} materials have frequently been used because chemical modification of the norepinephrine (NE) or epinephrine (E) structure is not necessary prior to analysis. Because its sensitivity is comparable to that of a radioenzymatic assay [11,12] and may exceed that of gas chromatography–mass spectrometry [13,14], many investigators have preferred the application of HPLC–ED. This method is also relatively inexpensive.

The objective of our study was to provide the simultaneous analysis of NE and E in platelet-rich plasma and serotonin in platelet-poor plasma using HPLC–ED. This technique, using the newly improved 3-μm Phase-2 ODS column, permits the accelerated analysis of picogram quantities of catechol- and indoleamines in plasma. The apparent ease of carrying out such determinations is also suitable for routine laboratory analysis, where multiple samples are processed.

0378-4347/89/$03.50

EXPERIMENTAL

Chemicals

NE, E, dihydroxybenzylamine (DHBA), and sodium octyl sulfate (SOS) were purchased from Bioanalytical Systems (BAS) (West Lafayette, IN, U.S.A.). Acetonitrile (HPLC grade) was supplied by Aldrich (Milwaukee, WI, U.S.A.). Monochloroacetic acid (MCAA), 5-hydroxytryptamine creatine sulfate (serotonin), N-acetylserotonin, trichloroacetic acid, sodium metabisulfite, Na_2EDTA, perchloric acid, sodium hydroxide, disodium phosphate, and Tris base were all reagent grade and were obtained from Sigma (St. Louis, MO, U.S.A.).

Chromatography

All experiments were carried out using a BAS Model 400 catecholamine analyzer. This consisted of an LC-4B amperometric detector, a solvent delivery system with pulse dampener, a Rheodyne Model 7125 injector, and a column heater. A glassy carbon working electrode was set at 650 mV versus an Ag/AgCl reference electrode. The sensitivity of the electrochemical detector was maintained at 1.0 nA full scale. Separation was performed on a 100 mm × 3 mm I.D., 3-μm Phase-2 ODS (BAS). The temperature of the column was set at 26 °C, and the flow-rate was 1.0 ml/min. The signal was recorded on a Houston recorder; areas of the peaks were integrated with an Adalab Chromcard II (Adalab, Chicago, IL, U.S.A.) on an Apple IIe system.

The mobile phase (pH 3.0–3.2) consisted of 1 l deionized distilled water, 14 g MCAA, 4.7 g sodium hydroxide pellets, 0.75 g Na_2EDTA, 200 mg SOS, and 3–5% acetonitrile (v/v). The solvent mixture was filtered through a 0.45-μm Millipore filter and degassed by spanging with helium prior to use. A Sonicor ultrasound bath (Copiague, NY, U.S.A.) was used to eliminate air bubbles that might interfere with the assay.

Analytical procedure

Standard solution of NE (140 ng/ml), E (45 ng/ml) and DHBA (100 ng/ml) were prepared in 0.1 *M* perchloric acid. The actual concentration of NE and E added to plasma samples were calculated as free base. All solutions were stored at −70 °C and prepared freshly every four weeks. Conditions for the quantitation of catecholamines are similar to those described by BAS (LCEL Application Note No. 14). The flow-rate for the present report was 1.0 ml/min, and the injection volume was 25 μl.

An initial 5-ml sample of whole blood was withdrawn from the femoral artery of anesthetized dog or domestic swine, and 10 ml were collected immediately and transferred into heparinized polycarbonate tubes containing approximately 1 mg sodium metabisulfite to prevent oxidation of amines [5]. The platelet-rich plasma was obtained by centrifuging the whole blood at 200 *g* for

10 min at 4°C. Samples were frozen at −70°C until time of assay. The remaining plasma was centrifuged at 1300 *g* to obtain platelet-poor plasma for the serotonin assay.

Catecholamine assay

After thawing, 1-ml samples were prepared for assay with addition of 25 μl DHBA (1 ng/ml final concentration) and 25 mg alumina; 500 μl of Tris–EDTA buffer (1.5 *M*, pH 8.6) were added to the assay tubes, and the contents vortex-mixed immediately for 5 min. The aqueous phase was discarded, and the alumina washed three times with deionized distilled water. Catecholamines were eluted from the alumina into 200 μl of 0.1 *M* perchloric acid. The mixture was located into a micro-centrifuge and the alumina–acid mixture was spun to dryness. The acidic extract containing catecholamines was then injected onto the column.

For each series of HPLC analyses, 1 ml of 0.1 *M* phosphate buffer (pH 7.0) containing known final concentrations of NE (1.8 ng/ml free base), E (0.63 ng/ml free base) and DHBA (1 ng/ml) was used as a control. The concentrations of NE and E were analyzed by integrating the areas under the identified peaks (Chromcard II) using an Apple IIe computer.

Serotonin assay

An appropriate amount of N-acetylserotonin (1 ng/ml final concentration) as an internal standard was added to 500 μl iced, thawed platelet-poor plasma. Trichloroacetic acid solution (125 μl of a 500 g/l stock) containing 50 m*M* sodium metabisulfite and 5 g/l Na_2EDTA was added prior to vortex-mixing for 10 min. The reaction vials were centrifuged at 6400 *g* for 6 min, and 25 μl of the resulting deproteinated supernatant were injected onto the 3-μm reversed-phase column. Conditions for the quantitation of serotonin are similar to those described above with minor alterations in the mobile phase composition. Stock buffer was prepared as follows: 14 g MCAA, 4.7 g sodium hydroxide, 0.75 g Na_2EDTA, 50 mg SOS, 3% acetonitrile (v/v), and 0.5% tetrahydrofuran (v/v). The effluent was again monitored electrochemically as above. N-Acetylserotonin was used as the internal standard for the quantitation of serotonin.

Precision of the assay

Catecholamines. Increasing concentrations of NE and E over the range 0.010–1.0 ng (total injected) were measured at 1.0 nA sensitivity. Each point on the calibration curve was averaged from two determinations. To determine the precision of the assay, twelve identical samples (1 ml each vial) were prepared from pooled canine/porcine plasma. Fresh platelet-rich plasma ($n=6$) was extracted after the preparation, and the remaining plasma samples ($n=6$) were frozen in a −70°C freezer and later assayed on two consecutive days. Precision was assessed as the intra- and inter-assay coefficients of variation

for NE and E using pooled plasma. Additional 6-ml pooled platelet-rich plasma samples having known concentration of NE and E (0.36 and 0.13 ng/ml free base final concentration, respectively) were used to evaluate the recovery of NE and E concentrations.

Serotonin. In an identical fashion to the NE and E experiments performed above, serotonin concentration was measured from platelet-poor plasma.

RESULTS

A linear relationship between the concentration of NE and E and percentage area (sample versus internal standard) was confirmed over the range 0.01–1.0 ng total injected onto the column. The line of best fit was determined by linear regression analysis. NE: $r^2=0.994$; $y=3.99+0.075x$; E: $r^2=0.992$; $y=3.45+0.0868x$. The chromatographic peaks were identified by retention times as compared to authentic compounds (NE, 1.98 min; E, 3.1 min; DHBA, 3.85 min). The detection limit for NE was 50 pg/ml and for E 10 pg/ml for a 1-ml sample of plasma, operating at a potential of 650 mV. Fig. 1 shows rep-

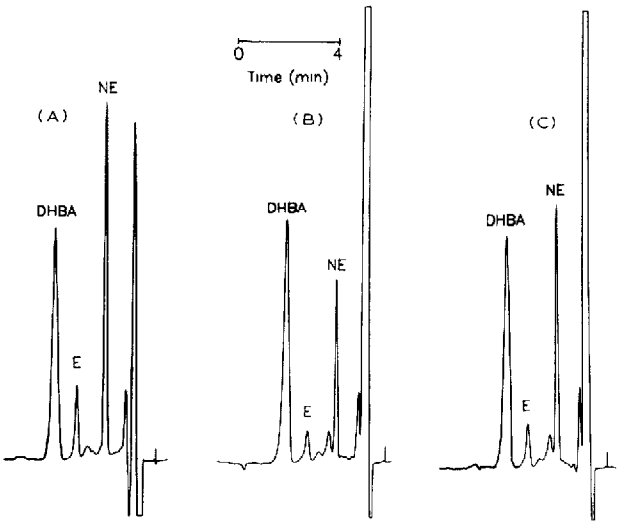

Fig. 1. Chromatograms of alumina extraction of catecholamines. (A) Chromatogram of a standard mixture containing 1.8 ng/ml NE free base, 0.63 ng/ml E free base, and 1 ng/ml DHBA. The retention times were 1.98 min for NE, 3.1 min for E, and 3.85 min for DHBA. (B) Typical chromatogram of an actual extract from pooled canine plasma. The concentrations assayed for NE and E were 0.65 and 0.025 ng/ml, respectively. (C) Chromatogram of plasma spiked with 0.36 ng/ml NE and 0.13 ng/ml E. Samples were prepared following the procedure outlined in the text. HPLC conditions are described in Experimental The flow-rate was held constant at 1.0 ml/min. Injection volume was 25 μl.

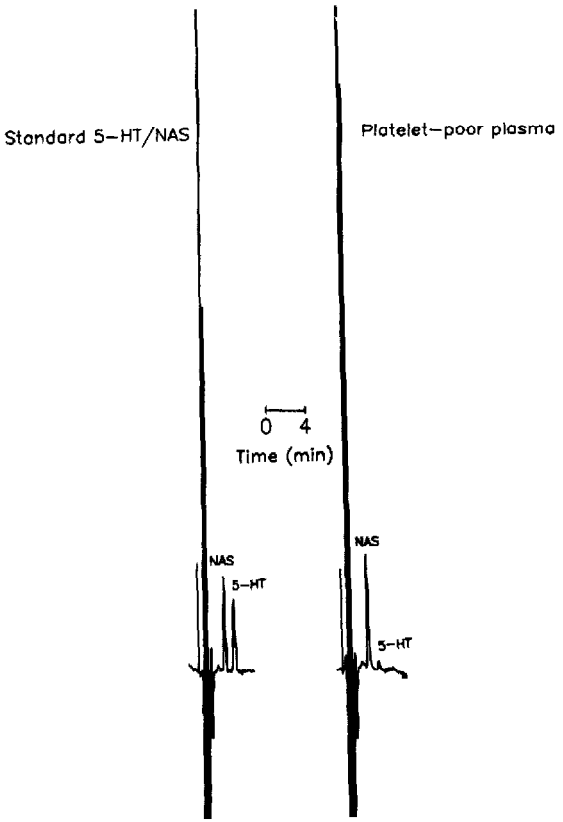

Fig. 2. Chromatograms of a standard mixture of serotonin (5-HT) and N-acetylserotonin (NAS) (1 ng/ml each, final concentration) and of a plasma sample worked up as described in the text. The concentration of serotonin was 100 pg/ml in the actual sample. A 50-μl aliquot of extracted sample was injected directly.

resentative chromatograms of standards of NE, E, and DHBA and of an actual plasma sample.

The intra-assay coefficient of variation was 4.19% for plasma NE concentrations exceeding 600 pg/ml and 6.4% for plasma E concentrations below 20 pg/ml. Additional serial determinations ($n=6$) of NE and E from frozen platelet-rich plasma were used to calculate the inter-assay variation for two consecutive days. The inter-assay coefficient of variation was 10.5% for NE (0.19 ± 0.02 ng/ml; mean $\pm$S.D.) and 12.3% for E (0.013 ± 0.0016 ng/ml; mean $\pm$S.D.).

To assess whether the extraction of NE and E from platelet-rich plasma was proportional to that known concentrations of NE and E added, we measured the recovery of the authentic compounds added to pooled plasma samples ($n=6$). Recoveries of known concentrations of NE and E added to platelet-

rich plasma were $92 \pm 2.23\%$ for NE concentrations of ≤ 1 ng/ml and $98.9 \pm 3.11\%$ for E concentrations of ≤ 0.20 ng/ml. These recoveries were corrected for the internal standard.

Recoveries of serotonin standards (corrected for internal standard) added to platelet-poor plasma were $>95\%$ for concentrations between 0.80 to 1.0 ng/ml. The limit of detection of serotonin in canine plasma was 0.50 ± 0.053 pg/ml. The intra-assay coefficient of variation was 1.92%. Fig. 2 shows representative chromatograms of an injection (50 μl) of a standard mixture and of an extracted plasma sample.

DISCUSSION

The HPLC–ED approach described in this report is an inexpensive method for the assay of two major classes of monoamines: catechol- and indoleamines. Improvement in chromatographic performance, selectivity, and sensitivity is achieved through the use of a 3-μm Phase-2 ODS reversed-phase column. In this technique, basal plasma concentrations of NE (≥ 0.5 ng/ml) and E (≥ 0.01 ng/ml) are easily measured in platelet-rich plasma; basal plasma concentrations of serotonin (0.8–1.0 ng/ml) also can be detected in platelet-poor plasma. Inter- and intra-assay coefficients of variation are relatively small, reflecting the reproducibility and precision of this method (see Results). A potential application of this technique is the simultaneous determination of multiple biogenic amines in single samples. Our modified procedure allows ninety determinations over an 8-h time period. Further, reagent costs are substantially less than for isotopic assay.

ACKNOWLEDGEMENTS

This work was supported by National Heart Lung and Blood Institute Grants HL-32495 and HL-35718. A. Leff is the recipient of National Heart Lung and Blood Institute Research Career Development Award HL-01398.

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