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# AUTOMATED MEASUREMENT OF CATECHOLAMINES IN URINE, PLASMA AND TISSUE HOMOGENATES BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY WITH FLUOROMETRIC DETECTION

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## SUMMARY

An automated catecholamine analyzer was assembled from a high-performance liquid chromatograph, an autosampler and an air segmented reactor equipped with a sensitive fluorometer. The trihydroxyindole technique was applied to the fluorometric reactor. Urine, plasma and brain homogenate were usually purified on an alumina column prior to high-performance liquid chromatography (HPLC). Centrifuged urine and a supernatant of brain homogenate can, however, be injected onto the HPLC column without further pre-treatment. The measurement of noradrenaline and adrenaline in plasma of a man at rest required 0.5–1 ml samples. Approximately 100 alumina eluates from urine can be treated per day when the analyzer is operated continuously.

## INTRODUCTION

In 1974, a new liquid chromatographic method for measuring catecholamines (CAs), based on the use of high-performance liquid chromatography (HPLC) with fluorometric detection, was presented[1]. Since then, routine procedures have been developed and implemented for determination of urinary CAs[2–6,14], metanephrines in urine[7] and CAs in plasma, cerebrospinal fluid and tissue homogenates[8], utilizing the trihydroxyindole (THI)[1–4,6–8] and ethylenediamine[5] methods. An automatic HPLC system for the measurement of urinary CAs was also assembled by means of an autosampler in combination with an AutoAnalyzer equipped with a fluorometer[6].

In this paper I describe the assay of urinary CAs with and without prepurification by the automated CA analyzer, and the application of the technique to measurements of CAs in plasma and brain homogenate.

## EXPERIMENTAL

### *Reagents*

CAs and alumina (neutral, Brockman activity grade 1) were obtained from Sigma (St. Louis, MO, U.S.A.), sodium octyl sulphate from Eastman (Rochester, NY, U.S.A.) and sodium 1-heptanesulphonate from Regis (Morton Grove, IL,

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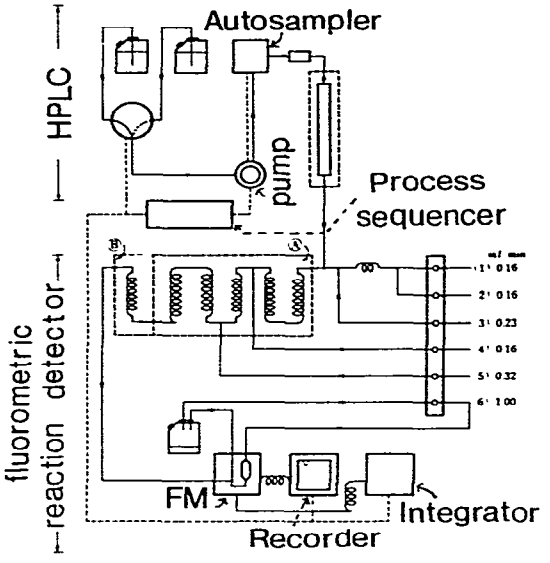



Fig. 1. Automated CA analyzer (THI method). Heating bath temperature: A, 30°C; B, 15°C (both 15°C for plasma). Reagents: 1 = 1 *M* phosphate buffer; 2 = 0.1% $K_3Fe(CN)_6$; 3 = air; 4 = 0.1% ascorbic acid; 5 = 4 *M* NaOH. FM = fluorometer.

U.S.A.). Other chemicals were reagent grade commercial materials. All chemicals were used without further purification.

*Apparatus*

Fig. 1 is a schematic presentation of the automated CA analyzer. It is comprised of a high-performance liquid chromatograph, an autosampler and an air segmented reactor equipped with a sensitive fluorometer. Unless otherwise stated, pumps, autosamplers, process sequencers, valves, gauges, dampers, connectors, etc., were from Kyowa-seimitsu (Tokyo, Japan) and proportioning pumps, glass coils, fittings, connectors, all tubing etc., were from Technicon (Chauncey, NY, U.S.A.). A Model 6000A solvent delivery system and a Model 710A WISP (Waters Assoc., Milford, MA, U.S.A.) were also used. Fluorometric measurements were made with a Model RF-510-LCA Shimadzu spectrofluorophotometer (excitation 420 nm, slit width 20 nm; emission 520 nm, slit width 40 nm). The fluorometer was equipped with a xenon lamp and a mirrored rectangular solid 120-$\mu$l flow cell. DuPont Zipax SCX, Hitachi gel 3011-C, Yanapak ODS-T and Waters $\mu$Bondapak $C_{18}$ were used as packing materials. Chromatographic conditions are described in the figure legends.

*Procedure*

Upon collection, urine was acidified to pH 3 with 6 *M* HCl and stored frozen at −20°C prior to analysis. To 10 ml of the acidified urine was added 1 ml of a 0.2 *M* solution of the disodium salt of EDTA. The pH was then adjusted to 8.5 by addition of 1 *M* NaOH. The solution was poured onto a column prepared from 0.5 g alumina and allowed to drain completely. The column was washed twice with 10 ml of distilled

water. The CAs were then eluted into a 10-ml test-tube using 5 ml of 0.5 *M* acetic acid. 10–200 μl of the eluent were injected onto the chromatographic column. Total CAs were determined by addition of 2 *M* HCl (5 ml) to 5 ml of the acidified urine in a 20-ml test-tube. After heating for 20 min on a water-bath at 85°C, the hydrolyzed urine was treated in the manner described above for the unhydrolyzed urine.

Blood was collected by antecubital venipuncture in a centrifuge tube containing the disodium salt of EDTA (1 mg per 1 ml blood). The plasma separated by centrifugation at 6000 *g* at 2°C for 30 min can either be analyzed immediately or stored frozen at −80°C. A 1-ml volume of the plasma was placed in a centrifuge tube and 20 μl glutathione-SH (60 mg/ml) and 500 μl of 1.5 *M* perchloric acid were added. The tube was shaken vigorously for 1 min, and the contents centrifuged at 18,000 *g* at 2°C for 30 min. The supernatant was then transferred to a centrifugal microfiltration device[9] and the pH of the deproteinized sample adjusted to 8.5 with 4 *M* and 1 *M* ammonium hydroxide. Fifty milligrams of alumina were added and the device was shaken for 10 min. The contents were filtered centrifugally at 3000 *g* for 10 min and the alumina was washed twice with 2.5 ml distilled water. The CAs were eluted into a 300-μl microvial for WISP using 100 μl of 1 *M* acetic acid. The wash and desorption steps are most conveniently carried out using the filtration device. A 90-μl volume of the eluate was injected onto the chromatographic column.

## RESULTS AND DISCUSSION

### *Automated measurement of CAs in human urine by HPLC with fluorometric detection using a Zipax SCX column*

The chromatograms of urinary free and total (conjugated and free) CAs separated by two-stepwise elution are shown in Fig. 2. The calibration curves for CAs were linear over the range of 0.5–50 ng for noradrenaline (NA) and adrenaline (A), and 0.02–0.5 μg for dopamine (DA). The coefficient of variation for each CA in intra-assay of an identical standard solution and of an identical alumina eluate from urine was 2.1% for NA, 0.7% for A, 2.2% for DA ($n = 5$) and 1.3% for NA, 2.2% for A,

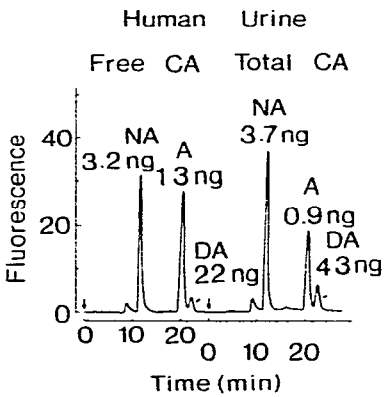


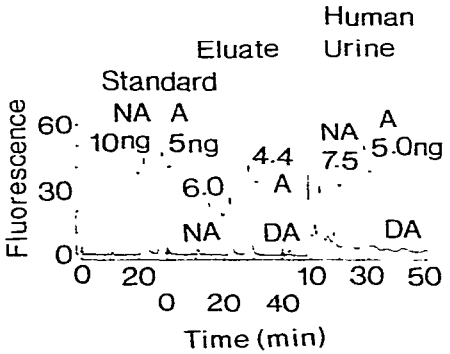

Fig. 2. Chromatograms of free and total CAs in human urine on a Zipax SCX column. Column: 1 m × 2.1 mm; temperature, 40°C. Mobile phase: 0.03 *M* $NaH_2PO_4$ to 0.1 *M* $NaH_2PO_4$ (pH 4.3) (two-stepwise elution); flow-rate, 0.8 ml/min. Samples: alumina eluates from urine specimens excreted for 0.3 min (free CAs) and 0.15 min (total CAs).

1.5% for DA ($n = 5$), respectively. The overall precision of the method was evaluated from multiple analyses on an identical specimen of urine. The coefficient of variation for each CA in five analyses was 0.5% for NA, 0.4% for A and 4.8% for DA. The overall recoveries of CAs added to urine were 95.8 ± 2.0% for NA, 97.5 ± 2.8% for A and 99.6 ± 2.6% for DA (mean ± S.D., $n = 10$). The sensitivity, reproducibility, precision and recovery were sufficient for the analysis of urinary CAs. If the automated CA analyzer is operated continuously, 70–100 samples per day can be treated by two-stepwise elution.

*Assay of urinary CAs without prepurification by the automated CA analyzer equipped with an Hitachi gel 3011-C column*

In the analyses of serotonin and 5-hydroxyindoleacetic acid in urine[10] and concentrated CAs in tissue homogenate[11] the centrifuged urine or the diluted supernatant can be injected on the HPLC column without further pretreatment, but not in the analysis of CAs, especially NA, in urine. CAs in urine can be now measured, without alumina extraction, by HPLC using an Hitachi gel 3011-C column. Fig. 3 shows the chromatograms (with and without alumina extraction) of free CAs in the urine of a man after playing tennis. In the latter direct method it is necessary to clean up the column with 0.075 *M* citrate buffer (pH 4.0) containing 10% acetonitrile for 15 min after each measurement to eliminate the materials which are retained on the column. The fluorescence of CAs under the same operating conditions was linear at least in the range of 1–20 ng for NA and 0.5–10 ng for A. The coefficient of variation for each CA in intra-assay of an identical standard solution was 1.1% for NA and 0.8% for A ($n = 5$).

The two methods with and without alumina extraction were run in parallel on ten human urine samples. The results are plotted in Fig. 4a for NA and in Fig. 4b for A. Plots of NA and A values (ng/ml) obtained by the indirect method *vs.* those obtained by the direct method fitted the straight lines $y = 1.12x + 0.24$ with a



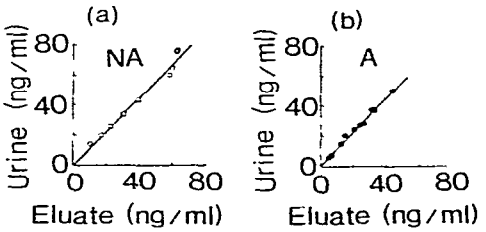




Fig. 3. Chromatograms of free CAs in human urine with and without alumina extraction on a Hitachi gel 3011-C column. Column: 20 cm × 4 mm; temperature, 55°C. Mobile phase: 0.044 *M* citric acid–0.031 *M* sodium citrate (pH 4.0); flow-rate, 0.8 ml/min. Sample size: 100 μl.

Fig. 4. Correlation of free CAs in human urine with and without alumina extraction on a Hitachi gel 3011-C column. Operating condition as in Fig. 3.

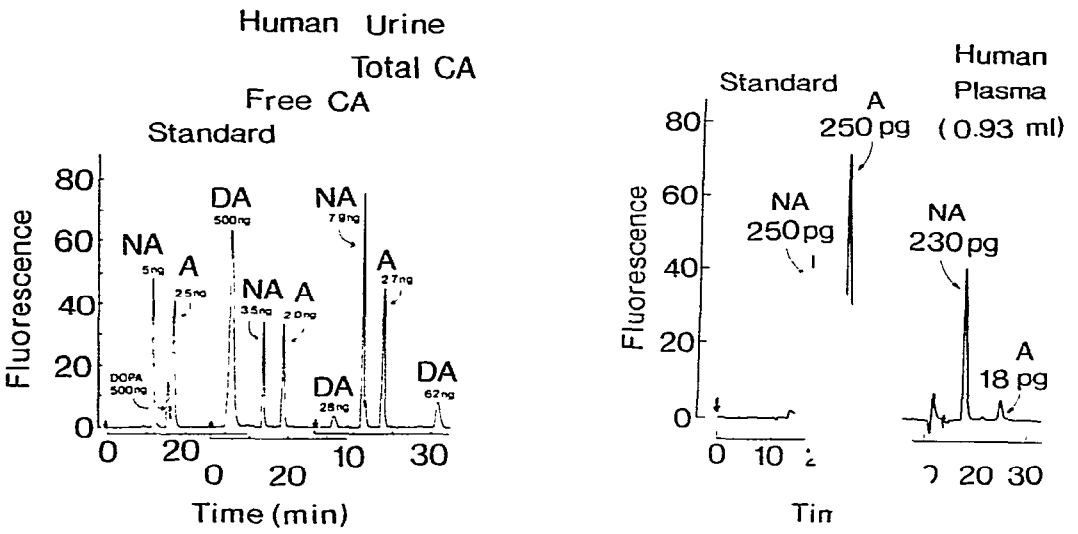



Fig. 5. Chromatograms of free and total CAs in human urine on a μBondapak $C_{18}$ column. Column: 30 cm × 4 mm; temperature, 20°C. Mobile phase: 0.065 *M* citric acid–0.035 *M* $Na_2HPO_4$–10 mg/l sodium octyl sulphate (pH 3.0); flow-rate, 1.2 ml/min. Samples: alumina eluate from urine specimens excreted for 0.3 min (free CAs) and 0.15 min (total CAs).

Fig. 6. Chromatograms of CAs in plasma on a Yanapak ODS-T column. Column: 25 cm × 4 mm; temperature, 20°C. Mobile phase: 0.1 *M* $KH_2PO_4$ + 100 mg/l sodium 1-heptanesulphonate (pH 3.1)–methanol (98:2); flow-rate, 0.6 ml/min. Sample: alumina eluate from plasma.

correlation coefficient of 0.983 and $y = 1.14x + 0.26$ with a correlation coefficient of 0.998, respectively. There are highly significant correlations between the values from the two methods ($p < 0.01$). The values for CAs in the alumina eluates are 88.6 ± 6.9% for NA and 87.0 ± 2.4% for A (mean ± S.D.) of those obtained directly from the urine samples. About 30 centrifuged urine samples per day can be measured by the direct method.

*Assay of CAs in human urine using μBondapak $C_{18}$*

Over the past years reversed-phase HPLC with chemically bonded octadecyl and octyl chains has become popular. The separation of CAs and related compounds has been carried out by soap chromatography or hydrophobic chromatography using these columns. Riggin and Kissinger[12] recently reported a highly effective procedure for simultaneous assay of urinary CAs by soap chromatography with electrochemical detection. It is necessary to use two-step extraction as sample preparation, *i.e.*, cation-exchange column extraction followed by an alumina column to eliminate acidic and neutral catechols which are retained on the $C_{18}$ column. Fig. 5 shows the chromatograms on a μBondapak $C_{18}$ column of free and total CAs in daytime urine from a woman. These samples were prepurified using only the alumina column. In the case of fluorometric detection using the THI method, the sample preparation is easier than in the case of electrochemical detection. Approximately 60 samples per day can be analyzed by the automated CA analyzer.

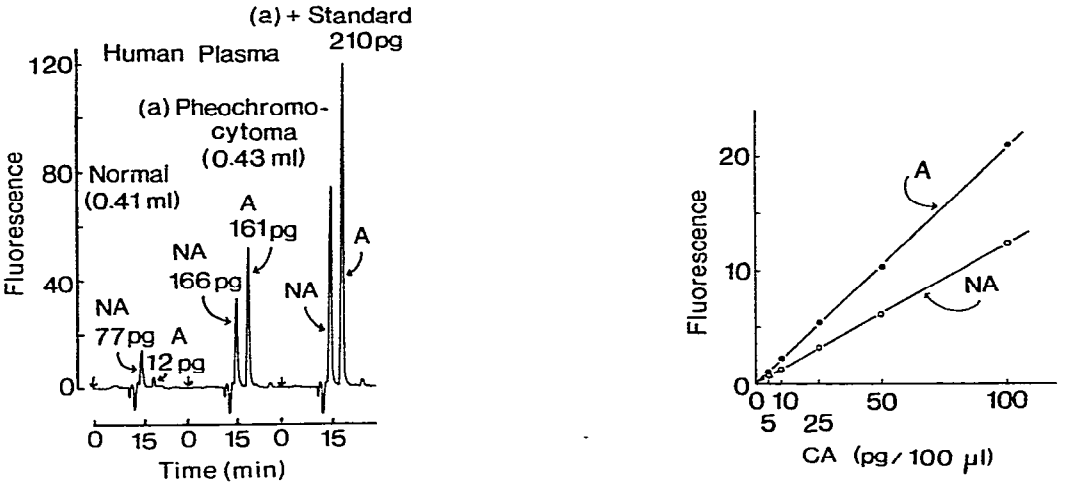



Fig. 7. Chromatograms of CAs in plasma on a Yanapak ODS-T column. Column as in Fig. 6. Mobile phase: 0.1 *M* $KH_2PO_4$ + 100 mg/l sodium 1-heptanesulpnonate (pH 3.1)–methanol (97:3); flow-rate, 0.7 ml/min. Samples: alumina eluates from plasma.

Fig. 8. Calibration curves for CAs on a Yanapak ODS-T column. Operating conditions, as in Fig. 6.

*Analysis of CAs in human plasma by use of Yanapak ODS-T*

CAs in human plasma were determined by ion-pair chromatography with a Yanapak ODS-T column as shown in Figs. 6 and 7. The calibration curves for CAs were linear over the range of 5–1000 pg for NA and A (Fig. 8). The coefficient of variation for each CA in intra-assay of an identical standard solution was 0.5% for NA and 0.8% for A ($n = 10$), respectively. The recoveries of CAs added to the supernatant were about 80% for NA and A. The measurement of NA and A in plasma of

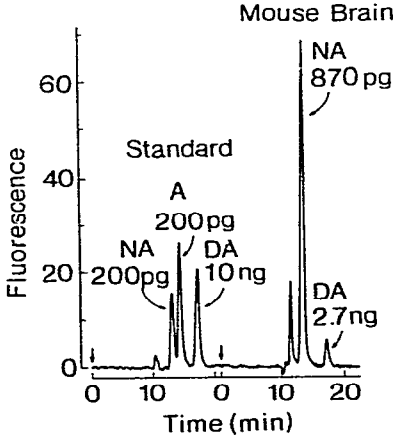



Fig. 9. Chromatograms of CAs in whole mouse brain on a Hitachi gel 3011-C column. Column: 15 cm × 4 mm; temperature, 55°C. Mobile phase: 0.15 *M* $KH_2PO_4$–0.15 *M* acetic acid (pH 3.3); flow-rate, 0.6 ml/min. Sample: supernatant from homogenate with 0.1 *N* $HClO_4$.

a man at rest was achieved for 0.5–1 ml samples. The measurement of only NA requires 0.1–0.3 ml plasma. Fig. 7 shows the chromatogram of CAs in plasma of a pheochromocytoma patient at rest. The CAs values in her plasma were 0.57 ng/ml for NA and 0.55 ng/ml for A. The CAs in the plasma of a healthy subject at rest (Fig. 7) were 0.27 ng/ml for NA and 42 pg/ml for A. At least 90 $\mu$l from the 100-$\mu$l alumina eluate in a vial can be injected in HPLC by use of an automatic sample injector (WISP 710A; Waters). About 70 samples per day can be analyzed by the automated CA analyzer equipped with the autosampler.

*Assay of CAs in mouse brain using Hitachi gel 3011-C*

The chromatograms of CAs in whole mouse brain are shown in Fig. 9. A 20-$\mu$l volume of the supernatant obtained from brain homogenate with 0.1 *M* perchloric acid was injected on the HPLC column. An alumina extraction procedure is necessary in order to detect the small amount of A in cerebral tissue. For the measurement of DA in plasma and brain parts, the chemical reactor based on ethylenediamine condensation or the *o*-phthalaldehyde method is preferable to that based on the THI method because of the stronger fluorescence obtained with the former reactor.

The use of air segmented reactors in HPLC has been reviewed by Frei and Scholten[13]. The automated CA analyzer with an air segmented reactor described here is useful for routine measurements of CAs in biological fluids and tissue homogenates.

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