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ASSAY OF FREE AND CONJUGATED CATECHOLAMINES BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY WITH ELECTROCHEMICAL DETECTION

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SUMMARY

A rapid and simple method for the analysis of free and conjugated catecholamines in body tissues and fluids is described. The free catecholamines were isolated by standard alumina procedures before and after hydrolysis of the conjugated compounds to free compounds by heating the samples in perchloric acid. Free catecholamines were then separated by high-performance liquid chromatography and detected by electrochemical detection. Conjugated compound was the difference between the total and free amount in each sample. This method was utilized to measure free and conjugated norepinephrine, epinephrine, and dopamine in human urine and rat adrenal gland, and to measure free and conjugated dopamine in rat whole brain and kidney.

INTRODUCTION

Conjugated dopamine (DA) has been found in greater concentrations than free DA in human plasma [1,2] and in both human and monkey urine and cerebrospinal fluid [3,4]. Much of the conjugated DA is thought to occur as sulfate esters [1,5,6]. In the course of developing methods to determine the physiological role of these DA sulfate esters, we found it necessary to have a simple assay available for the analysis of free and conjugated DA in various body tissues and fluids. This report details a method developed for this purpose, as modified to measure free and conjugated norepinephrine (NE) and epinephrine (EPI) in addition to free and conjugated DA. The free catecholamines (CA) are isolated by standard alumina procedures before and after hydrolysis of the conjugated CA to free CA. Free CA are then separated by reversed-phase paired-ion high-performance liquid chromatography (HPLC) and detected by electrochemical detection. We report values for free and

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conjugated DA, NE, and EPI in human urine and rat adrenal glands. Free and conjugated DA levels are reported for rat kidney and whole brain.

METHODS

Chromatographic apparatus

Two different liquid chromatographs were used. Each system was equipped with a stainless-steel column (25 cm × 4 mm) prepacked with 5-μm octadecyl silica (Bioanalytical Systems), a guard column (2 cm × 4.5 mm) packed with 40-μm C_{18}/Corasil (Waters Assoc., Milford, MA, U.S.A.), and an electrochemical detector (Bioanalytical Systems) consisting of a thin-layer CP-O carbon-paste working electrode and an LC-4 or LC-4A controller. One liquid chromatograph consisted of the following components purchased from Bioanalytical Systems: Waters M-45 pump, Rheodyne Model 7125 injection valve equipped with a 20- or 200-μl loop, and a Houston Instruments B-5000 strip chart recorder. Occasionally, a Constametric I pump (Laboratory Data Control) was used. A column of air connected in parallel with the solvent flow path served as a pressure dampener. The other liquid chromatograph was completely automated and consisted of the following components purchased from Waters Assoc.: Waters M-45 pump, WISP Model 710-B automatic sample injector, Model 720 system controller, and Model 730 data module.

Chromatographic conditions

The mobile phase utilized was a modification of that described by Davis and Kissinger [7] and consisted of 0.075 *M* phosphate buffer, pH 2.8, containing EDTA (1 m*M*), sodium octyl sulfate (30 mg/l), and methanol (5%). The mobile phase was passed through a 0.45-μm filter, degassed by sonication, and stirred continuously during use. The flow-rate varied depending on chromatographic conditions, but it was usually in the range of 1.0—2.4 ml/min. A gradient of increasing flow-rate was sometimes used to decrease retention time of late-eluting compounds. Hydrodynamic voltammograms for the CA under the conditions described above showed oxidation potential plateaus ranging from 0.60 to 0.75 V vs. and Ag/AgCl reference electrode. Consequently, the working electrode was maintained at an oxidation potential of 0.70 V for the analyses.

Assay of samples

Free and total catecholamines (CA) were measured in replicate aliquots of tissue homogenate supernatants or urine by a modified alumina adsorption procedure. Dihydroxybenzylamine (DHBA) and/or deoxyepinephrine (DOE) were used as internal standards. An aliquot of urine or tissue supernatant containing EDTA, sodium metabisulfite, and an appropriate amount of internal standard was hydrolyzed in 0.4 *N* perchloric acid at 100°C for 120 min to convert conjugated CA to free CA. The hydrolysis step was omitted for assay of free CA. The free CA were then isolated by standard alumina procedures described previously [4]. The samples were dissolved in mobile phase and injected into the HPLC system described above. Injection volume ranged from 5 to 100 μl. The ratios of endogenous DA, NE, or EPI to internal standard were

determined in each sample from the peak heights. The amount of each CA in each sample was calculated by inverse linear regression analysis of a standard curve constructed by addition of known amounts of each CA to internal standard and carried through the alumina adsorption and hydrolysis procedures. Conjugated DA, NE, or EPI was the difference between total and free amounts in duplicate aliquots.

Data analysis

Free and total amounts or concentrations of each CA were compared by one-tailed Student's *t*-test for paired observations [8]. A p value less than or equal to 0.05 was considered to be statistically significant. Conjugated CA was the difference between the total and free in each sample.

Collection and treatment of samples

Human urine was collected and prepared as described previously [9]. Female Wistar rats weighing 190—210 g were fasted overnight and sacrificed by decapitation. Brains and peripheral tissues were rapidly removed, dissected on ice if appropriate, frozen on dry ice, weighed, and immersed in tubes containing 0.4 *N* cold perchloric acid containing 2.5% sodium metabisulfite and saturated with EDTA. The tubes were stored at −80°C until use. The tubes were later thawed before use and appropriate amounts of internal standards were added to each tube. Each sample was then homogenized for 15 sec in a Brinkmann Polytron Homogenizer (Model PT 10-35) and centrifuged at 4°C at approximately 12,000 *g* for 20 min. Aliquots of the supernatant were then assayed as described above. Urine was centrifuged at 4°C at approximately 12,000 *g* for 20 min, aliquoted into tubes containing internal standard, and assayed as described above.

Determination of optimal hydrolysis conditions

Aliquots of human urine were assayed for free and total CA as described above, except that each aliquot was heated in 0.4 *N* perchloric acid for times ranging from 30 to 240 min.

RESULTS

HPLC assay

Calibration curves for standards carried through the extraction and hydrolysis procedures were found to be linear throughout the ranges used for each assay. These varied from 5—100 pmoles (for DA in kidneys) to 5—100 nmoles (for NE and EPI in adrenals). The hydrolysis procedure had no effect on the peak heights of NE, EPI, DHBA, DA, or DOE standards. Reagent blanks carried through the alumina extraction, with or without hydrolysis, did not produce any peaks corresponding to any of the CA peaks measured. The minimum detectable amount of NE, EPI, and DA (giving a peak-to-noise ratio of two) was approximately 0.25 pmoles injected onto the column.

The percentage coefficient of variation (C.V.) between and within assays was calculated for a human urine sample. The within assay C.V. was 3.7% or less for each of the free CA, and 5.7% or less for each of the hydrolyzed CA.

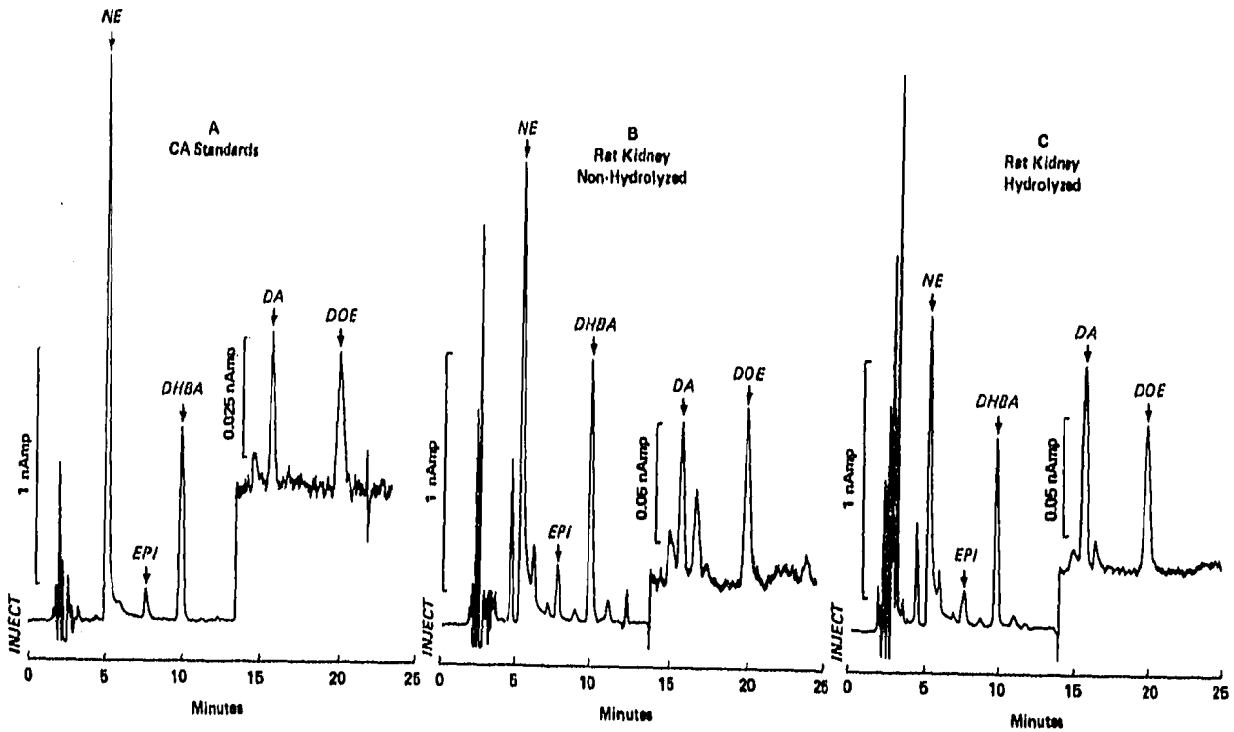

Fig. 1. Chromatograms of alumina extracts of catecholamine standard solution (A) and rat kidney homogenate supernatants before (B) and after (C) hydrolysis. There was no breakdown of any of the CA during the hydrolysis procedure. Note that total DA (hydrolyzed) is greater than free DA in this sample, indicating that substantial amounts of conjugated DA were present. HPLC conditions were as described in Methods. The flow-rate was held constant at 1.1 ml/min. Injection volume was 50 μl. The standard solution contained the following amounts of each CA per injection (assuming 100% recovery): NE, 200 pmoles; EPI, 10 pmoles; DHBA, 100 pmoles; DA, 5 pmoles, DOE, 20 pmoles.

The C.V. between assays was 8.8% or less for each of the free or hydrolyzed CA. Maximal recovery of total CA occurred in human urine after 120 min hydrolysis time. Consequently, 120 min was chosen as the hydrolysis time for all experiments.

Each of the CA of interest eluted from the HPLC column in a clearly defined peak. The capacity factors of NE, EPI, DHBA, DA, and DOE, plus those of other catechols which might interfere or be used as internal standards in these determinations, are given in Table I. None of the compounds injected resulted in interfering peaks in the analyses. Chromatograms of alumina extracts of CA standard solution and rat kidney supernatants before and after hydrolysis are shown in Fig. 1. The ratio of the DA peak height to either the DHBA or DOE internal standards is increased in the rat kidney after hydrolysis, indicating that substantial amounts of conjugated (total—free) DA are present.

TABLE I

CATECHOL RETENTION TIMES EXPRESSED AS CAPACITY FACTORS

n = 3 where replicates are indicated; n = 1 otherwise. 25 pmoles of each compound were injected. Capacity factor = (peak retention time — void volume time)/void volume time.

Compound	Capacity factor
Norepinephrine	1.60 ± 0.041
Epinephrine	2.91 ± 0.093
Dihydroxybenzylamine	4.05 ± 0.13
Dopamine	7.37 ± 0.29
Deoxyepinephrine	9.43 ± 0.36
3,4-Dihydroxyphenylglycol	1.19
Uric acid	1.34
3,4-Dihydroxymandelic acid	2.02
l-DOPA	3.66
3,4-Dihydroxyphenylacetic acid	10.5
Caffeic acid	11.3
α-Methyldopa	13.2
α-Methyldopamine	13.4
n-Acetyldopamine	14.1

Free and conjugated CA in rat tissue

The levels of free and conjugated NE, EPI, and DA in rat adrenal gland and free and conjugated DA in rat kidney and whole brain are shown in Fig. 2. Substantial amounts of conjugated DA were found in the kidney. The adrenal gland contained small but significant amounts of conjugated DA. Significant amounts of conjugated DA were not detected in the rat whole brain, nor were conjugated NE and EPI detected in the adrenal.

Because of the unexpected low levels or absence of conjugated DA in the rat adrenal gland and brain, several experiments were performed to verify these results. The assay was repeated six times on separate groups of four to eight rat adrenals, brains, and kidneys. The amount of conjugated DA detected

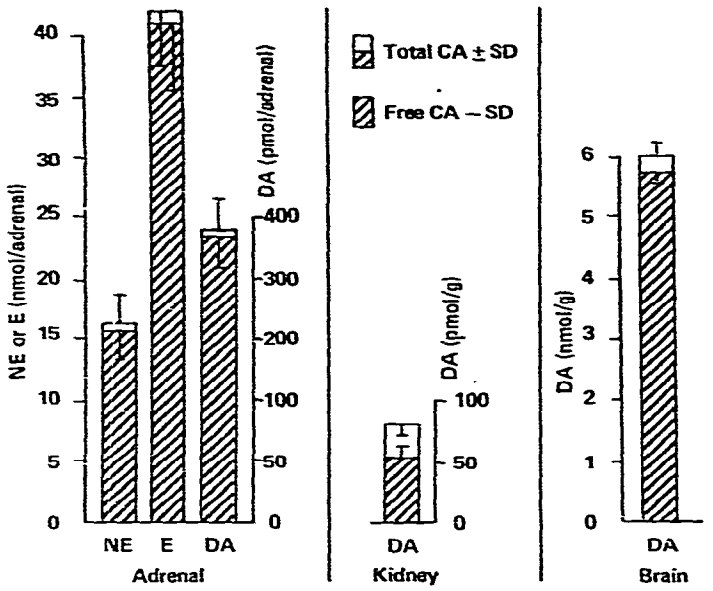

Fig. 2. Free and total catecholamines in rat tissue. Significant amounts of conjugated DA were found in the rat kidney but not in the whole brain. The adrenal contained small but significant amounts of conjugated DA, but not of conjugated NE or EPI. These results were verified in several separate experiments detailed in Results. $n = 6$ per group.

in the adrenals in these six repeated experiments ranged from 6.5 to 8.8% of total DA. Conjugated DA ranged from −4.5 to +3.4% of total DA present in rat brain. Repeated experiments on rat kidneys produced values for conjugated DA ranging from 26 to 34% of total DA.

The possibility that CA breakdown might be occurring during the hydrolysis procedure was considered and then eliminated by the fact that there were no differences in absolute peak heights of standards or sample CA peaks between hydrolyzed and non-hydrolyzed tubes. To test the hypothesis that incomplete hydrolysis might be occurring during the hydrolysis procedure, a mixture of dopamine-3-O-sulfate and dopamine-4-O-sulfate was added to rat adrenal glands. The adrenals were then assayed for free and conjugated DA, and complete hydrolysis of the dopamine sulfate to free DA was found to occur.

We also considered the possibility that premature hydrolysis might be occurring during the tissue disruption procedure in 0.4 *N* perchloric acid. To test this hypothesis, one of each pair of rat adrenals or kidneys was homogenized in 0.4 *N* perchloric acid (the routine procedure) and the other was homogenized in 1 *N* acetic acid. They were then assayed for free and conjugated CA as described in the methods section. If hydrolysis were occurring during homogenization in 0.4 *N* perchloric acid, then the free NE, EPI, or DA determined in the tissues homogenized in perchloric acid should be greater than that in the contralateral tissue homogenized in acetic acid. Results, shown in Table II, indicate that there were no differences in any of the free or total CA measured due to the homogenization medium.

One additional experiment was conducted to identify more conclusively the

TABLE II

EFFECT OF HOMOGENIZATION MEDIUM ON FREE AND TOTAL DOPAMINE IN RAT KIDNEYS AND ADRENALS

n = 6 per group; S.D. in parentheses.

	Kidney (pmoles/g)		Adrenal (pmoles/adrenal)	
	Perchloric acid	Acetic acid	Perchloric acid	Acetic acid
Free DA	52.4 (8.1)	55.6 (9.6)	159 (24)	157 (34)
Total DA	79.0 (8.9)	78.4 (7.8)	169 (31)	163 (32)
Percentage conjugated	34.3 (15)	29.5 (6.4)	5.32 (4.7)	4.31 (6.1)

peaks of interest in the chromatograms from rat tissues. Hydrodynamic voltammograms were generated over a portion of the useful oxidation potential range for standards of DA, NE, and EPI and compared with that portion of the chromatogram from rat adrenals thought to contain each of these CA. Since the curves for the standard and the compound from the adrenal were in good agreement (Fig. 3), we concluded that the peaks in the adrenals were correctly identified [10].

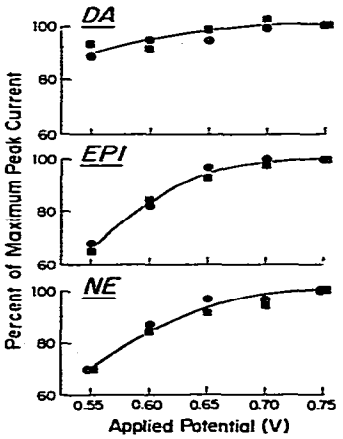

Fig. 3. Hydrodynamic voltammograms of NE, EPI, and DA in standard solutions (•) and rat adrenal gland (▪) alumina extracts. The close correlation in the responses to increasing oxidation potential strongly suggests that the peaks are correctly identified in the alumina extracts from the rat adrenal gland. HPLC conditions were as described in Methods.

Free and conjugated CA in human urine

Table III shows the mean daily urinary excretion of free and conjugated NE, EPI, and DA in the six subjects studied. Excretion of each of these CA in the conjugated form accounted for more than 50% of their total daily excretion. Conjugated DA accounted for 66.8 ± 7.7% of the total daily DA excretion as determined by HPLC in the present study. In an earlier study using

TABLE III

FREE AND CONJUGATED CATECHOLAMINES IN HUMAN URINE DETERMINED BY HPLC

n = 6, S.D. in parentheses.

	Excretion rate (nmoles/day)			Percentage conjugated
	Free	Conjugated	Total	
NE	653 (230)	727 (418)	1380 (623)	50.4 (7.4)
EPI	421 (139)	693 (594)	1114 (703)	55.9 (14)
DA	1161 (500)	2671 (1571)	3832 (2025)	66.8 (7.7)

a gas chromatographic—mass spectrometric method described elsewhere [4], we found that conjugated DA accounted for 61.5 ± 2.9% of the total daily DA excretion in four other normal human subjects (Elchisak and Ebert, unpublished). We have previously shown that dopamine-3-O-sulfate accounted for 73.1% of the conjugated DA excretion in four of these six subjects [9].

DISCUSSION

The HPLC assay for free and conjugated catecholamines reported in this paper has been in routine use in our laboratory for over one year. It is a rapid, sensitive, and relatively simple procedure for the analysis of free and conjugated catecholamines in most tissues and body fluids. The assay detailed here should be useful in future studies concerning the distribution of conjugated catecholamines in various body tissues and fluids. We are currently using this method to survey tissues and fluids of various species for the occurrence of conjugated DA. Since we are primarily interested in the distribution and functions of DA-3-O-sulfate and DA-4-O-sulfate in the body, we then examine those tissues and fluids in which conjugated DA occurs for the occurrence of the sulfated compounds. This procedure has been described elsewhere [9].

None of the possible interfering compounds listed in Table I, which might be carried through the alumina extraction procedure, actually interfered with the determination of the catecholamines of interest in the present study. However, the mobile phase must be carefully "fine tuned" to prevent dihydroxyphenylglycol, uric acid, and dihydroxymandelic acid from interfering with the NE peak. Varying amounts of methanol and sodium octyl sulfate can be added to the mobile phase, depending on column status, to eliminate interferences. We have most recently utilized a mobile phase containing 3% methanol and 40 mg/ml sodium octyl sulfate. Interference from uric acid can also be minimized, if necessary, by adding uricase to the sample mixture during the alumina adsorption step. This enzyme oxidizes uric acid to allantoin, a compound which is not electrochemically active and thus will not interfere with any of the catecholamine determinations. Interference from these compounds, in our hands, has only been a problem when an HPLC column which had been in constant use for approximately six months was used for the analyses.

The percentages of total NE, EPI, and DA excreted in human urine as the conjugated compound were found to be greater than 50% for each compound in the present study by the method described. These results are in good agreement with those found by other investigators using acid hydrolysis coupled with fluorometric [11,12], radioenzymatic [2,13], and HPLC [14] assay methods. These results clearly indicate that conjugation is a significant metabolic pathway for catecholamines in humans.

Significant amounts of conjugated DA in the whole rat brain were repeatedly not detected in the present study. To our knowledge, the occurrence of conjugated DA in whole rat brain has not previously been investigated. The levels of free DA in the whole rat brain found in the present investigation are in good agreement with those reported by other investigators using fluorometric [15], radioenymatic [16], and HPLC [17,18] assay methods. It is possible that conjugated DA could be present in discrete rat brain areas and not be detected in the whole brain because of effects of dilution. Recently, Buu et al. [19] have reported that DA-sulfate accounts for 31% of the total DA in rat hypothalamus, 12% in the hippocampus, and only 1% in the striatum. The distribution of conjugated DA, and specifically DA-sulfate, in the brains of various species should be further investigated. Correlation of DA-sulfate levels with the activity of the enzymes responsible for the formation of this compound and its possible in vivo hydrolysis to free DA should provide an insight into the function of this compound, if any, in the mammalian brain.

Small but significant amounts of conjugated DA were detected in the rat adrenal gland in the present study. Conjugated NE or EPI were not detected. To our knowledge, conjugation of CA has not been previously studied in rat adrenal. The effects of various stresses on the synthesis or utilization of conjugated DA remain to be evaluated. It is interesting to note, however, that administration of dexamethasone, a synthetic adrenocortical steroid, produced a five-fold increase in the activity of rat kidney phenolsulfotransferase [20], the enzyme responsible for the synthesis of DA-sulfate [21].

Conjugated DA accounted for approximately 30% of the total DA present in rat kidney in the present investigations. This is in contrast to the findings of Kuchel et al. [22], who reported that essentially all of the DA in rat kidney occurred as conjugated DA. It is difficult to reconcile these differences. Methodological differences between our assay and theirs cannot account for this discrepancy, since the results we obtained for human urine agree well with the results obtained by this group [2]. No details concerning the treatment of the rats were given by Kuchel's group, so discrepancies due to age, whether or not the animals were fasted, and the method of sacrifice cannot be compared. It is possible that one or more of these factors differed between the two studies, and that this might help account for the differences observed.

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