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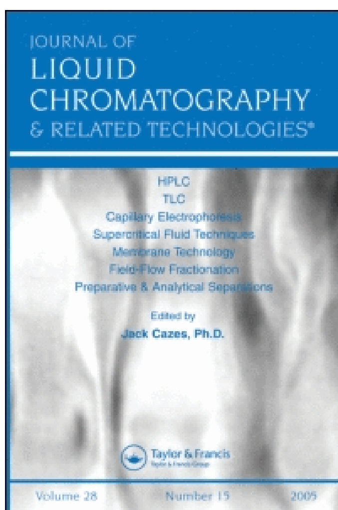

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MEASUREMENT OF CONJUGATED CATECHOLAMINES IN HUMAN PLASMA. A LOW COST HPLC-ECD METHOD

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ABSTRACT

We present an HPLC assay with electrochemical detection of conjugated catecholamine levels in human plasma. This method allows the direct assessment of the conjugated catecholamine values without the subtraction of the free catecholamine amounts: the assay is performed in the residual supernatant after the extraction (and the assay) of free catecholamines.
The results are comparable with the classic enzymatic and acid-heat hydrolysis methods (which yield the sum of free and conjugated catecholamine concentrations). This technique shows good reproducibility (within-run and between-run CVs < 8% in the physiological range), and sensitivity (< 20 pg per each deconjugated catecholamine per extract). This method allows about 30 low cost determinations of plasma free and conjugated catecholamines to be done in a working day.

INTRODUCTION

Conjugated catecholamines (CA) are an important fraction of total CA in human plasma (1, 2). It has

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been widely accepted that sulfoconjugation is an important metabolic pathway for the inactivation of some catechols. Further, conjugated CA were considered as metabolic end-products devoid of any effect. Conjugation of CA is heterogeneous, CA being conjugated with either sulfate or glucuronide groups (2). It has been shown that dopamine (DA) sulfate may serve as a substrate for the synthesis of both norepinephrine (NE) and epinephrine (Epi) (3). Finally, conjugated CA (chiefly sulfated) represent excretion products, but they may also constitute a form of CA storage for further retransformation into the free form, through an arylsulfatase system (4). Thus, in order to study the physiology and the pathophysiology of CA conjugation it is necessary to determine conjugated CA levels of endogenous CA. Several methods have been developed for the assay of both sulfo- and glucuro-conjugated CA. Most included a separate assay of free and free plus conjugated CA (1, 5 - 7). Others did not (8).In this study we present an HPLC method in which conjugated CA were determined by acid hydrolysis of the residual supernatant after the adsorption of free CA onto alumina. This method allows the direct determination of both free and conjugated CA in the same sample without calculations. Comparisons were made among the present method and others involving enzymatic hydrolysis of

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both sulfo- and glucuro-conjugated CA. In particular, sulfatase method (which yields the sum of free and sulfated CA), Beta glucuronidase method (free plus glucuronated CA), and acid heat hydrolysis method (free plus sulfated plus glucuronated CA), were investigated.

MATERIALS

Chemicals. All chemicals were of analytical or "HPLC" grade. NE, Epi, dihydroxybenzylamine (DHBA, internal standard), DA, alumina type WA-4, sulfatase type VI, beta glucuronidase type IX, sodium metabisulfite, sodium phosphate, sodium acetate, sodium dodecylsulfate, and Tris buffer were all from Sigma Chemical Co., St.Louis, MO. Acetonitrile LiChrosolv, phosphoric, hydrochloric and perchloric acids, and EDTA were all from Merck, Darmstadt, FRG. Water was purified in a Milli-Q apparatus (15-18 Mohm, Millipore Corp., Bedford, MA). All solutions were filtered by a solvent clarification kit (0.22 µm, Millipore Corp.). pH was measured at room temperature.

Instrumentation. For HPLC we used a system comprising a Model 510 pump (Waters, Milford, MA), a Model 7125 high pressure injection valve fitted with a 50 µL sample loop (Rheodyne, Cotati, CA) and a Model 5100A electrochemical detector (ESA, Bedford, MA) consisting of a series of three electrodes (Model 5011 and 5021 cells), working in the oxidation-reduction mode.

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The analytical column used was a stainless steel Supelcosil LC-18-DB, 7,5 cm x 4.6 mm (i.d.), prepacked with 3 µm ODS (Supelco Inc., Bellefonte, PA), protected by a precolumn Supelguard LC-18-DB, 2 cm x 4.6 mm (i.d.), also from Supelco.

The elution profiles were integrated by an LCD CI-10 B (Milton-Roy, Riviera Beach, FL), and displayed by a Sekonics plotter.

Mobile phase. The eluent consisted of an 84:16 (by vol) mixture of 50 mmol/L sodium phosphate, 50 mmol/L sodium acetate, and acetonitrile and also contained 0.6 mmol of sodium dodecylsulfate and 0.6 mmol of EDTA per liter. The final pH was adjusted to 3.10 with concentrated (850 g/L) ortophosphoric acid. The flow rate was 1.1 mL/min. The pressure on the column was 1750 psi. The solution was degassed by sonication (Model 250 Branson Sonic Power Co., Danbury, CT). The column was equilibrated with the mobile phase at least 6 h before use. The potentials applied were: +0.30 V (first electrode), +0.06 V (second), and -0.30 V (third). Output was monitored from the third electrode.

METHODS

Biochemical procedures. From pooled plasma 72 aliquots (2 mL each) were assayed for free CA as previously described onto alumina batch (9). The 72 supernatants

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(final volume 2 mL residual plasma plus 1 mL Tris buffer 1.5 mol/L, after alumina adsorption of free CA) were processed as follows: 8 were assayed again for free CA in order to determine if the extraction procedure was able to clear entirely free CA and DHBA; 32 underwent the enzymatic hydrolysis by sulfatase (each was added with 100 µL of 5 mol/L perchloric acid to adjust the final pH to 8.00, with 500 pg of DHBA, and with 1 U of type VI sulfatase; all were then incubated for 1 h at 37·C); 32 underwent the acid-heat hydrolysis (each was added with 1 mL of 5 mol/L perchloric acid, and with 500 pg of DHBA, vortexed, and centrifuged at 2500 x g for 10 min; the supernatant was then transferred in another tube and incubated for 10 min at 95·C).

32 aliquots (2 mL each), also from the same pooled plasma, directly underwent the sulfatase hydrolysis (as above but without the addition of perchloric acid); 32 aliquots underwent the Beta glucuronidase hydrolysis (2 mL of pooled plasma plus 1000 U Beta glucuronidase type IX, plus 500 pg of DHBA, incubated for 1 h at 37·C); and 32 underwent the acid-heat hydrolysis by the addition of 1 mL of 5 mol/L perchloric acid (these last 32 aliquots were added with 500 pg of DHBA and processed with the aforementioned acid-heat hydrolysis). After the acid-heat hydrolysis procedure, the aliquots were added with 2 mL of Tris-HCl buffer (5 mol/L, pH

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8.60) to adjust the final pH to 8.20 - 8.00. Each aliquot from various procedures was assayed for the deconjugated CA as previously described (9).

Finally, 20 aliquots (50 µL) of the plasma pool underwent a radioenzymatic assay of sulfated CA (6).

RESULTS

The electrochemical response was linear from the detection limits up to 20 ng/mL (r>0.996 for NE, Epi, DHBA, and DA). The detection limits (signal/noise ratio = 3) were: 10 pg per 50 µL of extracts for NE and 16 pg for Epi and DA (deconjugated either by enzymatic or by acid-heat procedure). The within-run CV was < 5% over 500 pg/mL and < 7% over 100 pg/mL of deconjugated CA. The between-run CV was < 7.5%, calculated respect to the peak height for the internal standard during five consecutive weeks (total n = 100). The chromatographic separations were completed in 12 min (lower separation times were hampered by unknown peaks). Analytical recovery was studied for every procedure of hydrolysis by addition of known amounts of CA standards in plasma aliquots and the comparison with the peak height yielded by the direct column injection of the same amount: [(peak height for plasma + standard) - (peak height for plasma)]/(peak height for standard directly injected). The mean (SD) ratios were: NE 0.60 (0.10),

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Table 1. Mean and SEM values (pg/mL) of free and conjugated CA levels yielded by different deconjugation procedures.

Group/n	NE	(SEM)	Epi	(SEM)	DA	(SEM)
A/72	367.23	(5.15)	66.25	(2.07)	173.40	(3.31)
B/32	417.92	(11.59)	387.36	(11.72)	974.04	(18.87)
C/32	1031.78	(23.15)	1381.48	(21.76)	2311.34	(19.97)
D/32	892.45	(7.33)	3118.63	(41.59)	2010.32	(25.35)
E/32	1176.35	(15.60)	3960.80	(48.44)	2197.89	(21.66)
F/32	361.50	(10.41)	1764.18	(29.36)	348.20	(16.67)
G/20	835.52	(13.53)	248.69	(7.48)	2976.73	(54.11)
H/08	Below Sensitivity Limits					

Groups. A: free CA. B: sulfated CA (sulfatase-treated supernatants). C: free plus sulfated CA (sulfatase-treated plasma). D: conjugated CA (acid-heat-treated supernatants). E: free plus conjugated CA (acid-heat-treated plasma, 1 mL perchloric acid). F: free plus glucuroconjugated CA (Beta glucuronidase-treated plasma). G: sulfated CA (sulfatase-treated plasma, by radioenzymatic assay). H: supernatants assayed again for free CA and DHBA (DHBA undetectable).

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Epi 0.68 (0.06), DA 0.51 (0.08), DHBA 0.48 (0.09) for enzymatic sulfatase procedure; NE 0.52 (0.09), Epi 0.58 (0.11), DA 0.42 (0.12), DHBA 0.33 (0.10) for enzymatic glucuronidase procedure; NE 0.64 (0.08), Epi 0.76 (0.08), DA 0.56 (0.06), DHBA 0.52 (0.05) for acid-heat procedures. Table 1 shows the results of the analysis of pooled plasma.

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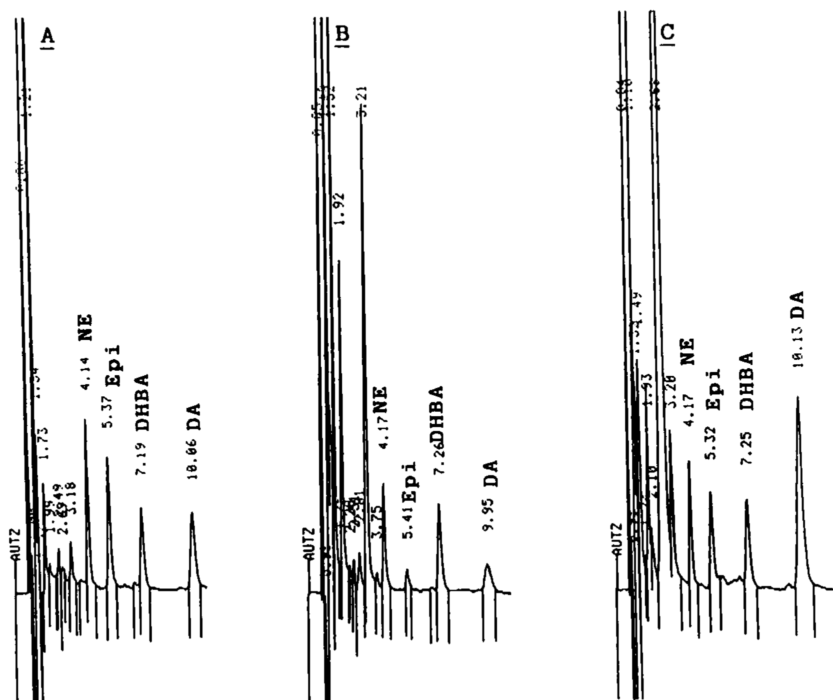

Figure 1. A) elution profile of a mixture of standards (1000 pg each). B) profile of free CA (NE 308 pg/mL, Epi 74 pg/mL, DA 169 pg/mL). C) profile of sulfoconjugated CA (supernatant incubated with sulfatase after free CA extraction: NE 411 pg/mL, Epi 401 pg/mL, DA 989 pg/mL). DHBA amount is 500 pg in each run.

Figures 1 and 2 show elution profiles of standards, free, and conjugated CA.

DISCUSSION

Previous methodological reports about the assay of plasma levels of conjugated CA enabled the determination of the sum of conjugated (sulfated and/or

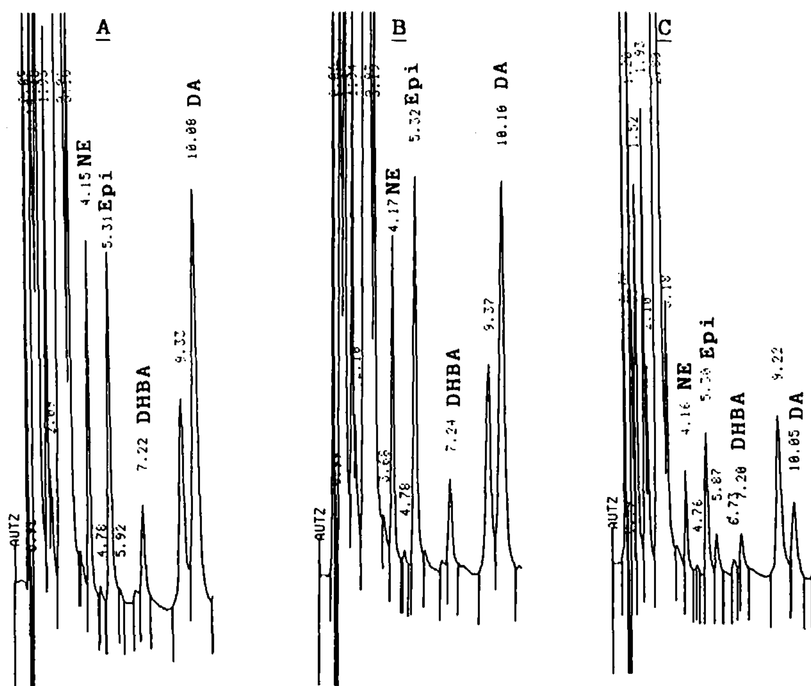

Figure 2. A) elution profile of conjugated plus free CA (plasma treated by acid-heat hydrolysis: NE 1124 pg/mL, Epi 3176 pg/mL, DA 2178 pg/mL). B) profile of conjugated CA (supernatant treated with the acid-heat hydrolysis after free CA extraction: NE 815 pg/mL, Epi 3492 pg/mL, DA 2190 pg/mL). C) profile of glucuronated plus free CA (plasma incubated with Beta glucuronidase: NE 400 pg/mL, Epi 1656 pg/mL, DA 397 pg/mL). DHBA amount is 500 pg in each run.

glucuronated) and free CA. Subtraction of free CA concentrations from the sum offered the value of conjugates (1,2,5-7). In the present study the possibility of a direct assay of conjugated CA after the determination of the free levels in the same sam-

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ple, is studied. We analyzed different hydrolysis techniques described in previous literature (1,5,6). Sulfatase hydrolysis in residual plasma seems insufficient, owing to the underestimation of all sulfated CA levels. This may be due to suboptimal incubation medium, pH, or ion concentrations. Enzymatic deconjugation of both sulfo- and glucuro- conjugated CA in plasma yielded values comparable with the acid-heat deconjugation (which enables the determination of the sum of sulfo- and glucuro-conjugated CA) in plasma, too. Beta glucuronidase method yields low recovery vs other methods.

The results obtained in residual supernatant, after free CA extraction, with the acid-heat procedure were comparable to those obtained in untreated plasma (NE 892, Epi 4119, DA 2010 versus NE 809, Epi 3895, DA 2025 - values in pg/mL) with the same procedure (after the subtraction of the free values). The values obtained following the HPLC technique were not comparable with those obtained by radioenzymatic assay for sulfated CA, probably owing to an insufficient resolution of the TLC step in the latter (10). In fact the sum of sulfated Epi and sulfated DA is comparable in the two assay (3453 vs 3226 pg/mL). Finally, the assay of conjugated CA in residual supernatants after free CA determination allows to minimize the amount of blood samples (5 mL

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respect to 10) with respect to the needs of a double determination of free and free plus conjugated CA. This method shows sufficient validity and reliability; the amount of samples analyzed per day may be as high as 30 including the extraction steps. The cost per sample is about 9 US$ (determination of both free and conjugated CA). It may be useful both in research and in routine analysis.

NOTE: in spite of a recovery rate below 100 % for free CA assay (9), we obtained undetectable levels of free CA when supernatants were assayed again for free CA after alumina adsorption. A possible answer may be that CA are lost in part during the various steps following alumina challenge, being the first supernatant free of CA content.

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