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Note

Analytical differences between the determination of plasma catecholamines by liquid chromatography with electrochemical detection and by radioenzymatic assay

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The use of radioenzymatic assays to measure catecholamines and the more recent development of liquid chromatographic (LC) methods has led to a number of studies in which plasma measurements of catecholamines by the two techniques have been compared [1—6]. Although generally good agreement between the two methods has been reported [1—4], some studies have shown differences in measurements by the two techniques. In one study plasma norepinephrine (NE) concentrations were systematically higher by the radioenzymatic assay while epinephrine (E) concentrations showed variable higher or lower values depending on the concentration range [5]. In another study comparing a large number of different radioenzymatic and LC assays from different laboratories, wide-ranging inter-laboratory variations for plasma measurements were found for both NE and E values [6]. It was suggested that more rigorous methods of assay validation were required such as comparison with an established reference method. The present study describes the results of a comparison made between an established radioenzymatic assay and an LC method using electrochemical detection.

EXPERIMENTAL

Sample collection and handling

Blood was collected via an intravenous cannula and stored on ice in heparinized tubes before centrifugation and separation of plasma which was stored in

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two aliquots at −70°C prior to analysis. Thawed plasma samples were recentrifuged to remove particulate protein before assay by both radioenzymatic and LC assays. Subjects from whom blood samples were taken included alcoholics studied during and after withdrawal and normal control subjects. Multiple blood samples were taken from subjects in supine and standing positions and from some subjects also before and during isometric handgrip exercise.

Liquid chromatography with electrochemical detection

Liquid chromatography with electrochemical detection was based on the reversed-phase method described by Goldstein et al. [3]. Samples (40 μl) of the alumina extract or standard solution were injected onto a Waters Nova-Pak C_{18} reversed-phase column (150 × 3.9 mm, particle size 4 μm) by way of a Rheodyne Model 7125 sample injector and quantitated using a Waters Model 410 electrochemical detector with glassy carbon electrode set at a potential of +0.72 V vs. Ag/AgCl, and the sensitivity of the detector set at 1 nA. The mobile phase (6.8 g/l sodium acetate buffer at pH 4.8 containing 100 mg/l EDTA, 1 g/l heptanesulfonic acid and 7–8% acetonitrile) was recycled through the system at a flow-rate of 0.8 ml/min using a Waters Model M510 solvent delivery system.

Radioenzymatic assay

The radioenzymatic assay was based on the method described by Peuler and Johnson [7]. The method was essentially unchanged except for a halving of the amounts of tritiated S-adenosylmethionine in the incubation mixture and the use of chloroform—ethanol—70% ethylamine (16:3:2) during thin-layer chromatography.

Validation of assays

The radioenzymatic and LC assays were validated by testing of linearity for radiolabelling or for the electrochemical response in relation to catecholamine concentrations, examination of the recoveries of catecholamines added to plasma and assessment of intra-assay and inter-assay reproducibility. Radioenzymatic and LC assays of plasma samples were carried out in random order using catecholamine standard solutions from the same source. Four samples showing different analytical results by the two assays were reexamined to check for linearity of radiolabelling. The alumina extracts of four samples were also analyzed by radioenzymatic assay in addition to LC and radioenzymatic assay of the unextracted plasma. The significance of differences between analytical results obtained using LC and radioenzymatic assays was assessed by Wilcoxon's signed rank sum test for related samples.

RESULTS AND DISCUSSION

Complete baseline separation of dihydroxyphenylacetic acid, NE, E, dihydroxybenzylamine, dopamine (DA), N-methyldopamine and isoprenaline was obtained using the LC method (Fig. 1). The electrochemical response was linear over the concentration range 25—2000 pg. In plasma extracts, occasional

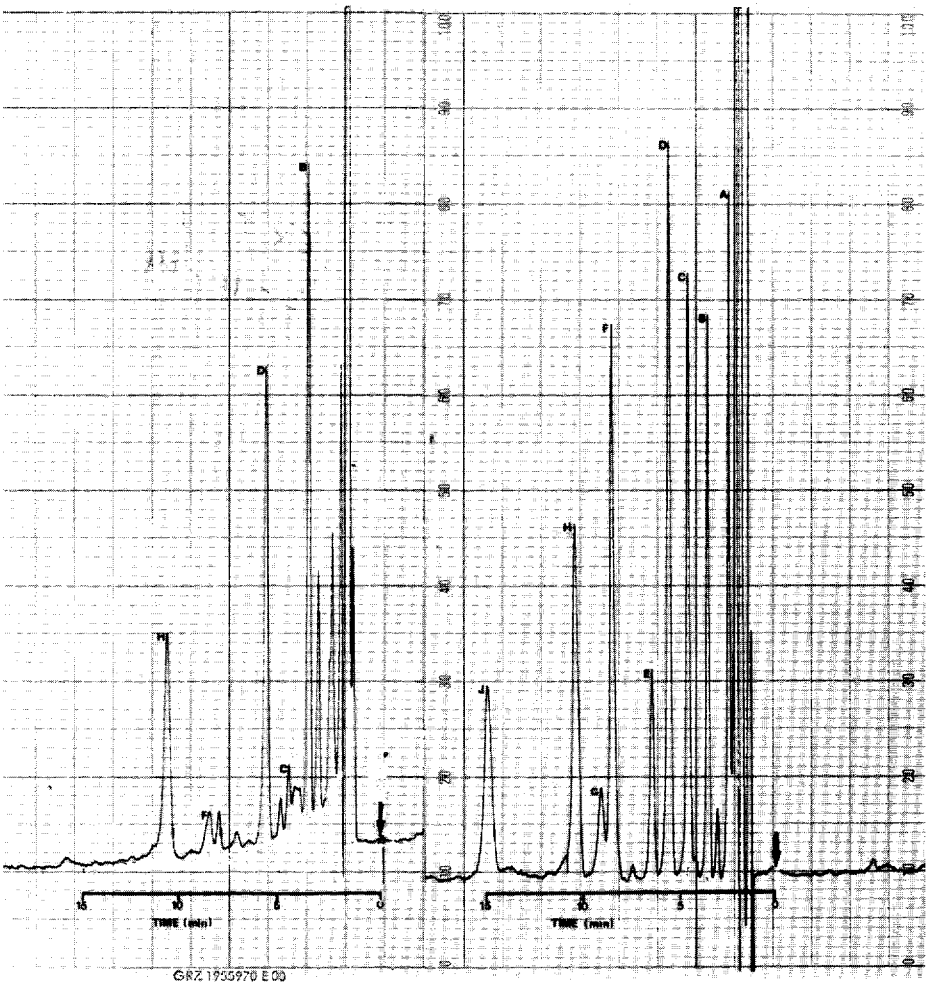

Fig. 1. Chromatograms obtained from the injection of a plasma-derived sample (left) and of a solution containing 400 pg each of dihydroxyphenylacetic acid (A), norepinephrine (B), epinephrine (C), dihydroxybenzylamine (D), normetanephrine (E), dopamine (F), metanephrine (G), N-methyldopamine (H) and isoprenaline (J).

interfering unknown peaks were found to co-chromatograph with DA, and the dihydroxyphenylacetic acid peak was often obscured by the solvent front. A peak of variable height, possibly dihydrocaffeic acid [8], was sometimes observed between E and dihydroxybenzylamine. Provided acetonitrile concentration in the mobile phase was kept below 8%, this was not found to be a source of interference as reported for a previous method [8, 9].

Inter-assay coefficients of variation for catecholamine determinations by the radioenzymatic assay were 3—16% for NE, 2—19% for E and 9—50% for DA, while by the LC method coefficients of variation were 7—9, 8—20 and 8—14% for NE, E and DA, respectively (Table I). For the radioenzymatic assay intra-assay coefficients of variation were 6.4% for NE and 7.6% for E, while by LC intra-assay coefficients of variation determined from thirteen duplicate alumina extracts were 1.5, 4.7 and 2.4% for NE, E and DA, respectively.

TABLE I

INTER-ASSAY COEFFICIENTS OF VARIATION (C.V.) FOR PLASMA CONCENTRATIONS OF NOREPINEPHRINE (NE), EPINEPHRINE (E) AND DOPAMINE (DA) DETERMINED USING LIQUID CHROMATOGRAPHIC AND RADIOENZYMATIC ASSAYS

Sample	Radioenzymatic assay			Liquid chromatography		
	n	Concentration (mean ± S.D.) (pg/ml)	C.V. (%)	*n*	Concentration (mean ± S.D.) (pg/ml)	C.V. (%)
1. NE	10	258 ± 28	11.0	No data		
E	10	149 ± 29	19.5			
2. NE	15	852 ± 88	10.3	No data		
E	15	230 ± 31	13.6			
3. NE	4	813 ± 127	15.7	13	463 ± 36	7.8
E	3	106 ± 3	2.3	13	87 ± 17	19.1
DA	4	221 ± 29	13.1	13	204 ± 25	12.1
4. NE	4	619 ± 32	5.2	5	501 ± 38	7.5
E	4	174 ± 9	5.3	5	180 ± 14	7.8
DA	4	100 ± 9	9.0	5	116 ± 16	14.0
5. NE	4	368 ± 10	2.6	5	239 ± 22	9.1
E	4	46 ± 2	5.0	5	56 ± 11	19.8
DA	4	28 ± 14	50.0	5	38 ± 5	7.9

Sensitivity of the radioenzymatic and LC assays varied between 5 and 20 pg of catecholamine measurable per ml of plasma.

Comparison of plasma catecholamine concentrations determined by LC and by radioenzymatic assay revealed a consistent analytical difference for the measurement of NE but not E (Fig. 2). Plasma NE concentrations for the 62 samples that were analyzed were significantly ($p < 0.001$) higher when measured by the radioenzymatic method [mean ± standard error of the mean (S.E.M.), 975 ± 71 pg/ml] compared with LC (584 ± 47 pg/ml). The extent of this analytical difference depended on the source of the plasma samples, some patients showing better agreement for plasma NE concentrations determined by the two techniques, than other patients with up to five-fold higher plasma NE concentrations determined by radioenzymatic compared with LC assays for some samples. Little difference was found between plasma E concentrations determined by LC (99 ± 11 pg/ml) and radioenzymatic assay (116 ± 12 pg/ml). Mean (± S.E.M.) plasma DA concentrations were 63 ± 4 pg/ml by LC and 73 ± 5 pg/ml by radioenzymatic assay.

A number of possible explanations for the observed analytical differences should be considered. One possibility could involve differential recoveries of catecholamines compared with the internal standard in the LC method, secondary to differential adsorption or desorption onto or from the alumina, differential degradation during extraction or the presence of a co-chromatographing peak interfering with the internal standard [8, 9]. These possibilities were discounted by using two internal standards the extraction efficiencies of which did not greatly differ from each other or from NE, E and DA. Mean extraction efficiencies during alumina extraction were 64% for dihydroxybenzylamine, 60% for N-methyldopamine, 58% for NE, 61% for E and 64% for DA.

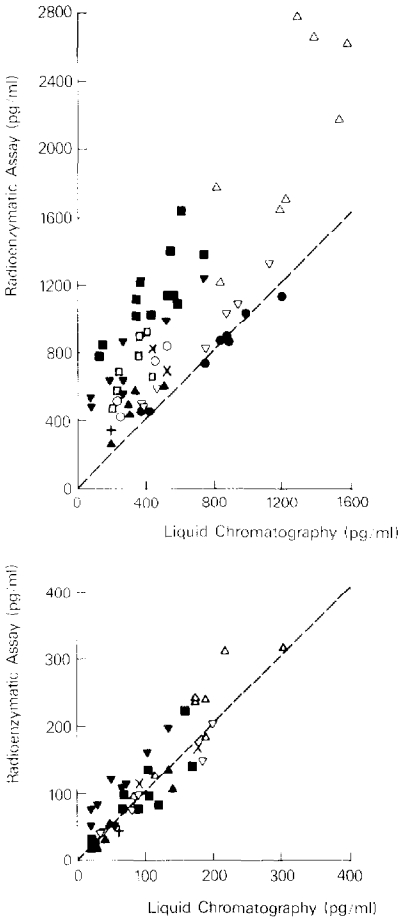

Fig. 2. Relationships between plasma norepinephrine (upper) and plasma epinephrine (lower) concentrations determined by liquid chromatography with electrochemical detection and by radioenzymatic assay. Samples from the same patients are represented by specific symbols. The crosses represent the three quality control samples repeatedly measured by both liquid chromatography and radioenzymatic assay (see Table I).

Another explanation for the observed analytical difference could involve non-linear enzymatic methylation by catechol-O-methyltransferase for some standard supplemented samples in the radioenzymatic assay [10]. This explanation was discounted by results showing that linearity was retained in the range 200—6000 pg/ml for plasma samples previously shown to give different analytical results by radioenzymatic assay and by LC. Also, recoveries of known amounts of catecholamines added to plasma samples before radioenzymatic assay were not greater than expected and did not differ from those obtained by the LC analysis of the same plasma sample. Mean recoveries of NE, E and DA for these samples were 87, 89 and 101%, respectively, by the radioenzymatic assay and 92, 87 and 117%, respectively, by LC.

A third explanation concerning the possible presence of contaminants in the catechol-O-methyltransferase enzyme preparation that may raise apparent NE values [10] is unlikely in view of the finding that the extent of the

analytical difference for NE values was dependent on the subject from whom the samples were taken.

The mean (± S.E.M.) values for NE concentrations for four samples determined using LC or radioenzymatic assay of the alumina extracts (corrected for extraction efficiency) and radioenzymatic assay of unextracted plasma were 387 ± 38, 382 ± 14 and 507 ± 34 pg/ml, respectively. This result together with the finding that the analytical difference was dependent on the subject from whom the samples were taken, suggests that a plasma constituent might be the source of the analytical difference. This could involve thin-layer cochromatography of labelled material with NE in the radioenzymatic assay or, alternatively, the presence in plasma of conjugated or bound forms of NE that are recognized by catechol-O-methyltransferase, but which may not be adsorbed onto alumina. Another possibility could involve a plasma constituent which diminishes the oxidative electrical potential generated by NE in the LC method. This, however, is unlikely in view of the similar values obtained for NE measured in alumina extracts by radioenzymatic assay and by LC.

In conclusion, the present results agree with those of another study showing that plasma NE concentrations may show higher values when measured by radioenzymatic assay compared with LC [5]. In addition, the present study indicates that this difference depends on the source of the plasma sample and that it is not due to differences in recovery, loss of linearity or assay contaminants but might be due to a variable level of a plasma constituent recognized as NE by the radioenzymatic assay but not by the LC method.

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