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Note

# Effect of haemolysis on high-performance liquid chromatography measurements of free plasma catecholamines

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The influence of haemolysis on the radioenzymatic assay of plasma catecholamines has been the subject of studies [1, 2] that have given conflicting results. In fact, haemolysis has been reported either to have no influence on the catechol-O-methyltransferase assay [1] or to be associated with a lower analytical recovery in a double-isotope radioenzymatic assay [2], possibly due to inhibition by intra-erythrocytic derivatives of enzymatic labelling of catecholamines.

Since high-performance liquid chromatography with electrochemical detection (HPLC—ED) is gaining wider acceptance [4, 5] and provides a tool for the direct measurement of catecholamines [3, 5], we investigated this question using an HPLC—ED technique employing a highly sensitive dual-electrode coulometric detector [6].

## EXPERIMENTAL

### *Reagents*

Norepinephrine (NE) bitartrate, epinephrine (E) bitartrate, 3,4-dihydroxybenzylamine (DHBA) hydrobromide, dopamine (DA) hydrochloride, aluminium oxide (activity grade super 1, neutral) and Tris—HCl (pH 8.6) were purchased from Sigma (St. Louis, MO, U.S.A.). Alumina was acid-activated according to the method of Anton and Sayre [7]. Sodium metabisulphite, disodium EDTA and citric acid were obtained from Merck (Darmstadt, F.R.G.). Diphenylborate—ethanolamine was from Aldrich (Milwaukee, WI, U.S.A.); tetraoctylammoniumbromide from Fluka (Buchs, Switzerland).

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Acetonitrile (HPLC grade), formic acid, acetic acid, *n*-heptane and *n*-octanol were purchased from Carlo Erba (Milan, Italy); octanesulphonic acid from Beckman Analytical (Milan, Italy).

*Equipment*

The HPLC—ED apparatus consisted of a Beckman 110 A pump (Beckman, Fullerton, CA, U.S.A.), a Waters WISP 710 B automatic injector (Waters Assoc., Milford, MA, U.S.A.), a Micro-Guard ODS-5 S column (Bio-Rad Labs., Segrate, Italy) and an Altex Ultrasphere ODS 5-$\mu$m (25 cm × 4.6 mm I.D.) reversed-phase analytical column.

The dual-electrode coulometric detector was ESA 5100 A with a 5010 analytical cell (ESA, Bedford, MA, U.S.A.).

A dual-pen chart recorder (OmniScribe D 5000; Houston Instruments, Austin, TX, U.S.A.) was used.

*Sample collection and handling*

Blood was drawn from seven recumbent healthy volunteers who were not allowed to smoke cigarettes, to drink caffeine-containing drinks or to take any medication. Extreme caution was used to avoid haemolysis. Blood was collected into prechilled polypropylene tubes containing 6% sodium EDTA, gently mixed by inversion and immediately put in ice.

*Hypotonic haemolysis*

The first four samples were divided into two series of five aliquots, each containing 5 ml of blood. The samples of one series were added to 200 $\mu$l of 0.1 *M* perchloric acid containing 60 ng of DHBA (internal standard) each; the other samples to 200 $\mu$l of 0.1 *M* perchloric acid containing 20 ng of NE, 40 ng of E and 60 ng of DHBA.

One of the five aliquots of each series was then mixed with 0.9% sodium chloride and the remaining four with saline solutions of decreasing osmolarity.

The per cent of haemolysis so induced was assessed by measuring the haemoglobin content in the corresponding plasma with a spectrophotometer at 540 nm and by referring to a standard curve of haemolysis.

*Freezing-induced haemolysis*

The latter three samples were spiked with 160 ng of NE, 320 ng of E and 480 ng of DHBA and then divided into two aliquots. One of them was quickly separated by centrifugation at 4°C and 2000 *g* for 10 min; the other was frozen at −20°C for 12 h in order to provoke haemolysis, thawed at 4°C and then centrifuged at 2000 *g* for 10 min.

*Isolation of catecholamines from plasma*

All measurements were performed in duplicate.

(a) Samples with hypotonic-induced haemolysis: 6 ml of plasma diluted with the different osmolarity saline solutions were treated in a polypropylene conical centrifuge tube with 200 $\mu$l of 0.1 *M* sodium metabisulphite, 200 $\mu$l of 0.3 *M* EDTA (pH 7.0), 800 $\mu$l of 0.5 *M* Tris—HCl (pH 8.6) and 40 mg of activated alumina. The tube was shaken for 30 min in a rotatory mixer.

Following 5 min of centrifugation at 1000 *g*, the supernatant was discarded and the alumina was washed three times with 0.03 *M* EDTA, followed by centrifugation each time, as above. The catecholamines were then eluted with 500 μl of 0.1 *M* perchloric acid. Following centrifugation at 1000 *g* for 5 min, a 20—200-μl sample of the supernatant was injected into the chromatograph.

(b) Samples with freezing-induced haemolysis: catecholamines were extracted from plasma of both aliquots according to the method of Smedes et al. [9] with minor modifications. The catecholamines were eluted with 250 μl of 0.08 *M* acetic acid and 20—50-μl aliquots were injected into the chromatograph.

*Chromatography*

The mobile phase was prepared by dissolving 210.14 mg of citric acid, 100 mg of disodium EDTA and 3.77 ml of 99% formic acid in 1 l of deionized (Milli Q, Millipore, Bedford, MA, U.S.A.) water. The pH was adjusted to 3.10 with 1 *M* potassium hydroxide. The solution was then filtered using a vacuum pump and 0.22-μm membrane filter (Millipore, GVWP 04700). A volume of 22 ml was discarded and replaced by acetonitrile. Finally, 80 mg of octanesulphonic acid were added. The solution was stirred, filtered again under vacuum through 0.22-μm aqueous membrane filters and degassed by sonication before use.

Formic acid was preferred to the commonly employed acetate, citrate or phosphate buffers. In addition to providing buffering capacity at a pH suitable for catecholamine analysis [4, 10], its volatility minimizes the damage to the chromatographic apparatus that may ensue if crystallization occurs upon evaporation [8].

Both the mobile phase and the column were thermostated in a water bath at 37°C. The flow-rate was 1.0 ml/min. The potentials of 0.30 and −0.10 V vs. ESA reference electrodes were applied at the first and second electrode, respectively, and the differential mode Det 1—Det 2 was used for generation of chromatograms. The sensitivity settings used were 10 × 100 (gain) and 10 mV (full scale value) on the ESA 5100 A and recorder, respectively.

*Statistical analysis*

Statistics were performed by non-parametric Wilcoxon and Friedman tests.

## RESULTS AND DISCUSSION

The effects of hypotonic haemolysis are shown in Fig. 1 and those of freezing-induced haemolysis in Table I. No statistical difference was observed between clear plasma and haemolysed samples. Therefore, in vitro haemolysis does not seem to affect the HPLC—ED assay of free plasma NE and E. This is in keeping with what has been reported for freezing-induced haemolysis with a classic catechol-O-methyltransferase (COMT) radioenzymatic assay [1], but it is in contrast with the observation of others [2] who reported a lower analytical recovery after hypotonic haemolysis using double-isotope COMT method. This has been attributed to partial inhibition of COMT-labelling of catecholamines by intra-erythrocytic S-adenosyl-L-methionine, non-specific methyltransferases and/or catecholamines [2].

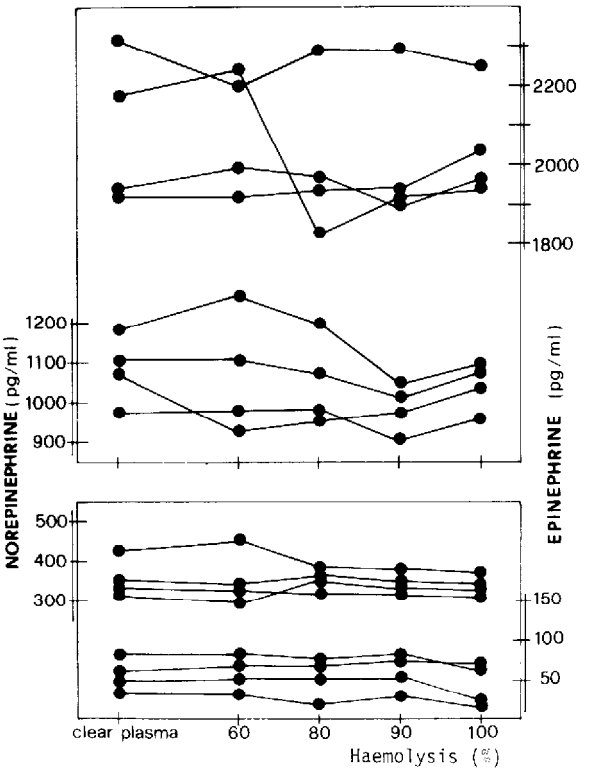


Fig. 1. Effect of hypotonic haemolysis on HPLC—ED measurements of free plasma norepinephrine and epinephrine in the nanogram (upper panel) and picogram (lower panel) ranges.

TABLE I

EFFECT OF FREEZING-INDUCED HAEMOLYSIS ON HPLC—ED MEASUREMENT OF FREE PLASMA NOREPINEPHRINE (NE) AND EPINEPHRINE (E) IN THE NANOGRAM RANGE

| Sample No. | Concentration (pg/ml) | | | |
|---|---|---|---|---|
| | Clear plasma | | Haemolysed plasma | |
| | NE | E | NE | E |
| 1 | 7435 | 14186 | 7382 | 13999 |
| 2 | 5944 | 10864 | 5716 | 10518 |
| 3 | 10530 | 13700 | 10746 | 13786 |

Since HPLC—ED is based on measurement of the current generated by a redox reaction occurring at the OH groups of catecholamines in an acidic solution [3, 5], it provides a tool for direct assay of these molecules. Our observation of lack of effect of haemolysis on these measurements provides evidence that free plasma catecholamines determined by HPLC—ED are unaffected by products of haemolysis and that catecholamines are not liberated by erythrocytes. Furthermore, it indirectly suggests that the aforementioned

explanation for the lower analytical recovery observed with the double-isotope COMT method may be correct.

Occasionally, we have observed a lower analytical recovery both in non-haemolysed and haemolysed samples. This was most probably due to a lower per cent of extraction of catecholamines from plasma since both NE and E and also the internal standard DHBA displayed the same behaviour. From this standpoint, the solvent extraction system [9] seems to provide a higher and more reproducible analytical recovery than the classic method employing acid-activated alumina [7]. As a consequence, although it seems that free plasma catecholamines can also be accurately measured in haemolysed samples by HPLC—ED, the use of internal standard, which allows one to make corrections for lower recovery during the extraction procedure, appears to be mandatory.

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