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Note

Assay of brain 3,4-dihydroxyphenylethyleneglycol (DHPG) levels by high-performance liquid chromatography with electrochemical detection

Factors affecting stability during sample preparation

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In recent years sensitive assays for 3,4-dihydroxyphenylethyleneglycol (DHPG), a major metabolite of noradrenaline (NA), have been developed using high-performance liquid chromatography (HPLC) with electrochemical detection (ED)[1,2]. These techniques require the extraction of catechols into strongly acid solutions prior to assay. However, no previous studies have determined whether free DHPG levels are stable under these conditions. The present study reports that free DHPG levels may rise substantially when extracts of tissue samples are kept in strongly acid solutions for longer than 12 h and that the rate of rise is temperature dependent. Evidence is presented that the rise is due to acid hydrolysis of conjugated DHPG recovered in the extraction procedure.

EXPERIMENTAL

Female Wistar-Kyoto rats (200–300 g) were stunned and decapitated. The brains were removed rapidly and homogenized immediately in 0.1 *M* perchloric acid (PCA) containing 0.05% EDTA, 0.05% sodium metabisulfite and dihydroxybenzylamine (DHBA) 100 ng/ml as the internal standard. Homogenates were centrifuged at 10,000 *g* for 8 min and aliquots (150–500 μl) adjusted to pH 8.6 by the addition of 1 *M* Tris (pH 8.6). Catechols were adsorbed onto 50 mg of alumina by mixing in the upper compartment of MF-1 microfuge tubes (Bioanalytical Systems) for 15 min. The alumina was retained on Whatman GF/B filters following centrifugation at 1000 *g* for 5 min and washed three times with 0.5 ml 0.1 *M* Tris (pH 8.6). Catechols were eluted by centrifuging 250 μl of 0.1 *M* PCA with 0.05% EDTA through the alumina. DHPG, NA, adrenaline (A), dopamine (DA) and 3,4-dihydroxyphenylalanine (DOPAC) were assayed simultaneously by HPLC-ED[2] using a WISP automatic injector for overnight assays.

Eluates from alumina of whole brain samples were pooled and divided into two aliquots, one kept at 4°C and the other at 28°C. Each aliquot was assayed in duplicate after 0, 2, 17, 24 and 48 h. The results, presented in Table I, showed that

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TABLE I

RISE IN FREE DHPG LEVELS WITH TIME

Eluates from alumina of pooled whole brain samples left at 4°C and 28°C for various times prior to assay. Values are the mean of two estimates which differed by less than 5%. Free DHPG levels rose significantly with time at both temperatures ($p < 0.001$). Temperature, independent of time had a significant influence on levels ($p < 0.001$), and there was a significant temperature × time interaction ($p < 0.001$, two-way analysis of variance).

Assay time (h)	*Free DHPG levels in whole brain (ng/g)*	
	4°C	*28°C*
0	23.7	23.7
2	22.9	24.4
12	25.6	31.9
24	28.3	36.3
48	30.7	39.4

free DHPG levels rose significantly over 24 h in both aliquots and that the rise was more marked at 28°C. Levels of the other catechols assayed did not change significantly. To investigate whether the rise in free DHPG was due to acid hydrolysis of conjugated DHPG, alumina eluates of brain samples were heated in sealed polycarbonate tubes at 95°C for 15 min to hydrolyse conjugated catechols[3]. Free DHPG levels increased from 15.0 ± 0.2 ng/g to 39.4 ± 2.9 ng/g (mean ± S.E., $n = 3$, $p < 0.005$, t test). Conjugated DHPG levels present in brain homogenates hydrolysed prior to alumina extraction accounted for 79% of the total metabolite present (18.1 ± 0.8 ng/g free, 89.9 ± 1.1 ng/g total, mean ± S.E.M., $n = 3$). These data indicate that a considerable proportion of conjugated DHPG is recovered in alumina extraction procedures and can undergo hydrolysis in 0.1 *M* PCA. Preliminary experiments suggested that acid hydrolysis of DHPG does not occur at pH levels greater than 1.0. However, adjusting the sample pH to more than 1.0 to retard hydrolysis of conjugated DHPG produced instability of the electrochemical detector.

To confirm the effect of temperature on the stability of DHPG levels in 0.1 *M* PCA, pooled alumina eluates of whole rat brain samples were incubated at 4, 22 and 37°C for 24 h. Levels following incubation at these temperatures were 9.5 ± 0.7, 9.9 ± 0.9 and 40.1 ± 3.5 ng/g respectively (mean ± S.E.M., $n = 4$). These differences were statistically significant ($p < 0.001$, one-way analysis of variance) and confirm that hydrolysis of conjugated DHPG is temperature dependent. The results also suggest that the rise after 24 h at 22°C was insignificant. To confirm this, alumina extracts of whole brain kept at 22°C were assayed in duplicate every 2 h for 12 h. There were no significant differences between any of these pairs of data the mean values of which ranged from 8.9 to 10.7 ng/g (pooled standard deviation 0.7, analysis of variance).

The assay of catechols by HPLC-ED with systems using automatic injectors is becoming popular, as they allow overnight processing of relatively large numbers of samples. In many cases these assay systems are maintained at room temperature. The present study has indicated that free DHPG in tissue samples may rise substantially when catechols are extracted into strongly acid solutions, and that the rise is

time and temperature dependent. However, levels appeared to remain relatively constant provided samples were kept at less than 25°C and assayed within 12 h.

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