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MODIFICATION OF THE RADIOENZYMATIC ASSAY FOR THE CATECHOLAMINES

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Abstract—There have been several reports describing the use of catechol-*O*-methyl transferase (COMT; EC 2.1.1.1) together with radioactively labelled *S*-adenosyl methionine (SAM) for the quantification of the catecholamines in biological material[1–4]. The procedure involves the enzymatic transfer of the radioactively labelled methyl group of SAM to the phenolic function at the 3-position of the catecholamine, the subsequent purification of the radioactively labelled product and its quantification by liquid scintillation counting.

While Nikodijevik *et al.* [1] used a highly purified COMT preparation for the determination of noradrenaline (NA) Cuello *et al.* [2] found this to be unnecessary in the quantification of dopamine (DA). The main disadvantage with the procedure of Cuello *et al.* [2] is the final stage in which purification of the radioactively labelled *O*-methylated products of the catecholamines is carried out by paper chromatography; we have found this to be both time consuming and also to result in loss of the amine derivatives. Fry and co-workers [3], subsequently improved the method by converting the *O*-methylated amines to their acetyl derivatives prior to their separation by paper chromatography. In order to facilitate the more rapid analysis of these catecholamines by the radio-enzymatic procedure we have modified the methods of Cuello [2] and Fry [3] to allow the separation and purification of the catecholamines using thin-layer chromatography.

MATERIALS AND METHODS

Water was redistilled in an all glass apparatus. All materials were Analar reagents (B.D.H. Chemicals Ltd., Poole, Dorset) except where stated and they were used without further purification. Acetic anhydride was redistilled from potassium hydroxide, the fraction boiling between 139° and 140° was collected and stored in an all glass container at 4° until required. The partially purified COMT preparation was obtained from rat liver, basically according to the method of Axelrod and Tomchick [4]. The activity of the enzyme preparation was between 300 and 500 nmoles product/mg protein/hr as determined by the method of McCaman [5].

S-Adenosyl [^{3}H]Me L-methionine (specific radioactivity about 8 Ci/mmole) was obtained from New England Nuclear, West Germany. LQD silica plates were obtained from Scientific Industries Ltd., Loughborough, Leicestershire. The solvent system was prepared by taking 30 ml of the upper organic phase from a mixture of toluene–methanol–water–ethyl acetate in the ratios 10:5:5:4, and adding to it 4 ml ethyl acetate, 2 ml methanol and 6.5 ml butan-2-one; the tank was also saturated with the aqueous phase from the first system by placing a beaker of this aqueous phase in the tank. Tissue samples were homogenised in 0.1 M perchloric acid containing 10 mg per cent disodium EDTA using an all glass homogeniser to give 10 per cent homogenates and the samples were centrifuged in a Beckman microfuge (type B) at room temperature for 90 sec.

The incubation mixture for the catecholamine assay was made up just before use and consisted of 50 μl 20 mM EGTA (adjusted to pH 7.2), 250 μl rat liver COMT preparation in 0.5 mM sodium phosphate buffer pH 7.0 (20 mg protein/ml), 150 μl [^{3}H]Me SAM (250 μCi/ml), 50 μl of a solution containing 16 mg/ml of pargyline in water containing 10% (v/v) 2-mercaptoethanol, 325 μl 1 M Tris base containing 3 mM magnesium chloride adjusted to pH 10.4.

To 25 μl of this incubation mixture in glass tubes (50 × 10 mm) on ice was added either 10 μl 0.1 M perchloric acid containing 10 mg per cent disodium EDTA as blanks, various concentrations of the catecholamines in 10 μl 0.1 M perchloric acid to provide the standard curve (normally 1 to 5 ng) or 10 μl of the tissue sample supernatant. In addition 1 ng of catecholamine standards in 2 μl of water were added to certain tissue samples to provide internal standards. All determinations were performed in duplicate. The contents of the tubes were mixed carefully and incubated at 37° for 40 min. The tubes were then removed and cooled to 0° and to each was added 30 μl of a mixture of 5 vols of 0.5 M borate buffer pH 10.0 and 1 volume of carrier methoxyamine mix containing 3-methoxytyramine,

metanephrine and normetanephrine each at a concentration of 330 μg/ml and 0.5 ml of toluene–isoamyl alcohol (3:2, v/v). The tubes were shaken vigorously on a vortex mixer for 30 sec and centrifuged to separate the phases.

An aliquot of the organic phase (400 μl) was transferred to 50 μl of 0.1 M hydrochloric acid and shaken vigorously for 30 sec; the samples were then centrifuged to separate the phases. The organic layer was then removed by aspiration and 40 μl of the acid phase was added to 150 μl water containing 3 μg of each of the methoxylated amines 3-methoxytyramine, metanephrine and normetanephrine. A small amount of sodium bicarbonate was added followed by 25 μl of acetic anhydride; further additions of sodium bicarbonate were made until the effervescence ceased. The acetylation procedure was then repeated. The samples were then extracted by shaking vigorously with 150 μl ethyl acetate for 1 min and the phases separated by centrifugation. An aliquot of the organic phase (normally 75 μl) was then placed on the origin of a 20 cm × 20 cm LQD silica plate and developed for 15 cm in the solvent system described above. The amines were visualised as blue-black spots after spraying the plate liberally with concentrated ammonia followed by a light spray with Folin–Ciocalteau reagent (diluted 1:2 with water). The spots were carefully removed from the plate while still damp and placed in counting vials; they were eluted overnight with 2 ml ethoxyethanol. Following the addition of 10 ml 0.4% butyl-PBD (2(4′-tert-butylphenyl)-5(4″-biphenylyl)-1,3,4 oxadiazole) in toluene, the radioactivity was estimated with liquid scintillation counting.

RESULTS AND DISCUSSION

LQD 19 channel silica plates were used in this work because of the facility with which these plates could be spotted and the spots removed after the chromatography had been completed. Several solvent systems were investigated in order to separate the derivatives formed from NA, DA and adrenaline. The solvent system which gave the best separation and the lowest blank values was modified from that described by Fry *et al.* [3] as described in Methods, the R_f values of the derivatives under these conditions were for NA 0.36–0.39, for adrenaline 0.45–0.49 and for DA 0.54–0.59. The blank values obtained in the assay were 50–90 d.p.m. for NA, 100–200 d.p.m. for DA and 30–60 d.p.m. for adrenaline which gave limits of sensitivity (2 × blank) of 40–100 pg for NA, 20–40 pg for DA and 10–30 pg for adrenaline. The recoveries obtained in the assay were 83.2 ± 8.2% for NA, 80.0 ± 9.5% for DA and 84.4 ± 4.6% for adrenaline (mean = S.E.M., $n = 5$).

In order to determine the possible error which the presence of one catecholamine had on the determination of the other two, 5000 pg of each of the amines in turn was carried through the procedure and the interference which resulted in the determination of the other two amines was calculated. These so-called 'overlap values' are shown in Table 1.

Table 1. 'Overlap errors' in the TLC of the amine derivatives

Amine added to sample	Overlap value for:-	Amount of overlap (pg)
NA	NA → Adr	100
	NA → DA	27
Adr	Adr → NA	60
	Adr → DA	5
DA	DA → Adr	180
	DA → NA	95

To separate samples were added 5 ng of each of the catecholamines and the assay carried out as described in the text. The radioactivity present in each of the amine bands was determined. Each of the overlap values is the mean of four separate analyses.

It can be seen that the overlap values are small and that in the normal working range of less than 2 ng of each of the amines per sample the resultant overlap figures would be below the limits of sensitivity for the assay of those amines.

The method described above is very sensitive and reproducible (normally better than 4% on duplicate determinations). The advantages of the modification reported here are that the chromatographic separation can be completed in approximately 1 hr with the result that determination of NA, DA and adrenaline content can be carried out in duplicate on twenty tissue samples together with the appropriate blanks, internal standards and standard curve in 1 day.

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