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A specific and sensitive microassay for hydroxyindoles in biological material—application for cerebral microvessels, brain, and pineal organ of the rat

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Serotonin (5-hydroxytryptamine; 5-HT) is an important neurotransmitter in the mammalian central nervous system. One of the most effective methods for measuring small amounts of serotonin involves conversion of serotonin to [^{3}H]melatonin [1]. In our experience, however, this radioenzymatic microassay for serotonin does not provide the sensitivity initially reported (50 pg, approximately 280 fmoles), mainly because the radioactive product ([^{3}H]melatonin) is not adequately resolved from other isotopic products, so that higher blanks and diminished sensitivity result. We have revised this assay to improve its sensitivity and reliability, by including unidimensional multiple-development thin-layer chromatography (UMD-TLC).

Materials and methods

Reagents were of analytical grade and were obtained commercially (Sigma Chemical Co., St. Louis, MO). Rat liver *N*-acetyltransferase (NAT: EC 2.3.1.5) and bovine hydroxyindole *O*-methyltransferase (HIOMT; EC 2.1.1.4) were prepared as described by Saavedra *et al* [1]; each microliter of NAT acylated 0.056 nmole of tryptamine in 15 min at 37°, and each microliter of HIOMT methylated 0.14 nmole of *N*-acetyl serotonin (NAS) in 15 min at 37°.

Precoated silica gel TLC plates (LK5D; Whatman, Inc., Clifton, NJ) were activated at 200° for 10 min immediately before use. Adenosyl-L-methionine-*S*-[methyl-^{3}H] (SAM; 5–11 Ci/mmole) and [^{3}H]melatonin (25 Ci/mmole) were purchased from New England Nuclear (Boston, MA).

Male Sprague–Dawley rats (Charles River Breeding Laboratories, Wilmington, MA) were maintained at an ambient temperature of 23 ± 2°, on a 12-hr light–dark cycle, and allowed access to food and water *ad lib*. Brain microvessels, free of contaminating blood elements, were prepared from individual rat brains by a previously described sucrose density-gradient method [2].

Serotonin assay. Tissue samples of microvessels (2–3 mg), brain (5–20 mg) and pineal (1–1.5 mg) were homogenized in 100, 200 or 400 μl of 4 M formic acid, respectively, with a 1-ml ground-glass homogenizer (Kontes Glass Co., Vineland, NJ). Ten microliters were removed for determination of protein content [3], while the remainder, along with an ethanol wash of equal volume, was collected in 1.5-ml plastic centrifuge tubes and centrifuged at 30,000 *g* for 30 min at 4°. Duplicate aliquots of the clear supernatant fluid (75 μl for microvessels, 150 μl for brain tissue, and 5–10 μl for pineal) were transferred to 16 × 100 mm tubes and dried *in vacuo*. The residue was dissolved in 50 μl of phosphate buffer (pH 7.9, 0.5 M) containing 5×10^{-6} M *m*-hydroxybenzylhydrazine and subjected to the following reactions.

Ten microliters of a 1.24-mM *S*-acetyl coenzyme A (AcCoA) solution was added to each of the tubes, which were then placed in a 37° water bath. The enzymatic reactions were begun by adding 10 μl NAT. After 30 min, each sample received 19 μl phosphate buffer, 2 μl (1 μCi, 0.147 nmole) [^{3}H]SAM, and 4 μl HIOMT. After an additional 15 min, the reaction was stopped by adding 0.5 ml of a boric acid buffer (pH 10.0, 0.5 M) containing 1 μg of unlabeled melatonin.

The melatonin was extracted quantitatively from the reaction mixture by vortexing each sample for 30 sec with 3 ml $CHCl_3$. Samples were spun for 10 min at 500 *g*, and the aqueous phase was aspirated and discarded. The organic phase was vortexed briefly with an equal volume of 1 mM HCl, which was removed similarly. Two milliliters of the organic phase was transferred to a fresh set of 13- × 100-mm test tubes and dried under a stream of nitrogen gas. The dried samples were dissolved in 75 μl of absolute ethanol and spotted on TLC plates. The TLC plates were developed by UMD ascending chromatography: the plates were developed to a height of 10 cm, allowed to dry for 5 min (maintained in the vertical position), and developed again a total of eight times with pure chloroform as the developing solvent [4]. The spots corresponding to authentic melatonin were visualized with short-wave ultraviolet light or with Ehrlich's reagent [5], scraped into 5-ml scintillation vials, eluted with 300 μl of absolute ethanol for 10 min, and counted in the presence of 3 ml toluene phosphor at an efficiency of approximately 32 per cent.

NAS assay. Samples assayed for NAS were treated similarly to those assayed for serotonin, except that NAT and AcCoA were omitted from the reaction mixture. Inasmuch as the brain contains minute amounts of NAS, this compound does not normally add to the amount of [^{3}H]melatonin formed. In the pineal, however, the radioactivity generated by endogenous NAS may be considerable and therefore must be subtracted from the total.

Results and discussion

Using conditions similar to those of Saavedra *et al*. [1], we found this newly developed assay to be linear between 125 fmoles and 4 pmoles of added serotonin or NAS in aqueous media (Fig. 1) or in the presence of tissue. When increasing amounts of serotonin were added to equal amounts of a microvessel homogenate, the isotopic melatonin formed remained linear over the range of concentrations tested (Fig. 1). Under these conditions, the recovery of exogenous serotonin was always more than 80 per cent, and the intra-assay coefficient of variation was less than 5 per cent.

To demonstrate the advantages of UMD-TLC, we compared samples chromatographed by UMD-TLC with those

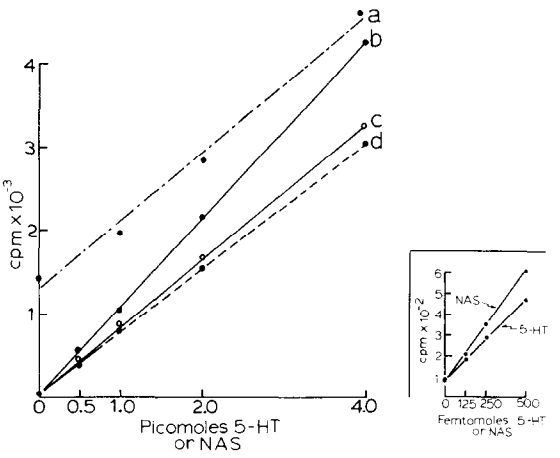

Fig. 1. Standard curves for serotonin and NAS. Key: (a) standard curve for serotonin in which the UMD-TLC step was omitted; (b) standard curve for NAS; (c) standard curve for serotonin; and (d) standard curve for serotonin generated by adding increasing amounts of serotonin to a homogenate of rat brain microvessels (the cpm due to endogenous serotonin has been subtracted). The insert in Fig. 1 is a standard curve for serotonin and for NAS, run in the femtomolar range.

extracted into chloroform alone. Known amounts of serotonin were added to a tissue homogenate and incubated as described previously. Duplicate samples were pooled, and equal aliquots were removed for either a simple chloroform extraction (i.e. the organic extract was dried in an 80° oven overnight and counted) or the same extraction and TLC. Blank values generated by TLC were more than six times lower than those resulting from the simple organic extraction (Fig. 1a).

To assess the specificity of our UMD-TLC procedure, we measured serotonin contents in the following samples: microvessels, microvessels plus a known amount of added serotonin, microvessels boiled in aqueous ammonia (to destroy the endogenous serotonin), or an aqueous sample of serotonin alone. The chloroform extract was spotted on TLC plates developed and cut into 2-mm segments from origin to solvent front. The solutes were eluted with ethanol and counted for tritium. A characteristic peak of radioactivity was seen at the location of authentic co-chromatographed melatonin in three samples (Fig. 2). Tissues boiled in aqueous ammonia contained minor amounts of background radioactivity at the melatonin R_f. Samples containing added serotonin and other components contained two peaks of radioactivity: one co-chromatographed with melatonin, and a second, larger peak at the solvent front. In tissue extracts prepared from microvessels, a third peak appeared, travelling only a short distance from the origin. Similar peaks of extraneous radioactivity, which we have not yet identified, were also detected when samples were extracted into toluene instead of chloroform.

Virtually all of the radioactive melatonin separated in the experiment shown in Fig. 2 was recovered in a 2-mm segment of the silica gel plate. Moreover, gas chromatography–mass spectrometry analysis [6] showed that this 2-mm segment was free of indoles other than melatonin.

Chloroform was used to extract radiolabeled melatonin from the reaction mixture because it is more volatile and dries faster than toluene, and because it extracts [^{3}H]melatonin more efficiently than does toluene. When 25,000 cpm of [^{3}H]melatonin were added to borate buffer and extracted with either chloroform or toluene, chloroform extracted more than 99 per cent of the indole whereas toluene extracted 91 per cent (unpublished observation).

We next applied this microassay to measure serotonin levels in microvessels obtained from a single rat brain; a previous anatomical study suggested its association with blood vessels in the brain [7]. We determined that rat brain microvessels contain 10 ± 2 pmoles serotonin/mg protein. Injection of the monoamine oxidase inhibitor pargyline (75 mg/kg, i.p.) 2 hr before decapitation elevated microvessel serotonin levels from 10 ± 2 to 17 ± 2 pmoles/mg protein ($P < 0.01$). The serotonin levels in 5–7 mg of right frontal cortex also rose, from 35 ± 3 to 55 ± 6 pmoles/mg protein ($P < 0.01$). The NAS contents of these tissues were below the limit of detection of the assay, which is consistent with earlier observations [8].

We also used the new method for quantitating both serotonin and NAS in a single rat pineal organ. It was possible to measure simultaneously serotonin and NAS in less than 15 per cent of a single pineal organ obtained during the light or dark period (Table 1). A highly significant, inverse daily variation was observed in the concentrations of these two indoles ($P < 0.001$). These findings agree with those previously published, where larger amounts of pineal homogenates were used [9].

The advantages of the present method are manifold: extremely small amounts of tissue can be assayed accurately for serotonin and NAS the identification of the radioactive product is unequivocal and the assay is simple and inexpensive to perform.

In summary, we increased the sensitivity and reliability of an existing radioenzymatic microassay for serotonin by coupling the procedure to a UMD-TLC procedure, which

Table 1. Levels of serotonin and NAS in rat pineal*

	Hydroxyindoles (ng/organ)	
	4:00 p.m.	4:00 a.m.
Serotonin	76.5 ± 7.6	25.1 ± 1.6†
NAS	0.8 ± 0.4	9.4 ± 1.2†

* Male Sprague–Dawley rats (500–550 g body wt) were killed at 4:00 p.m. or 4:00 a.m., and their pineals were assayed for serotonin and NAS, as described in Materials and Methods.

† $P < 0.001$, compared with tissues collected at 4:00 p.m.

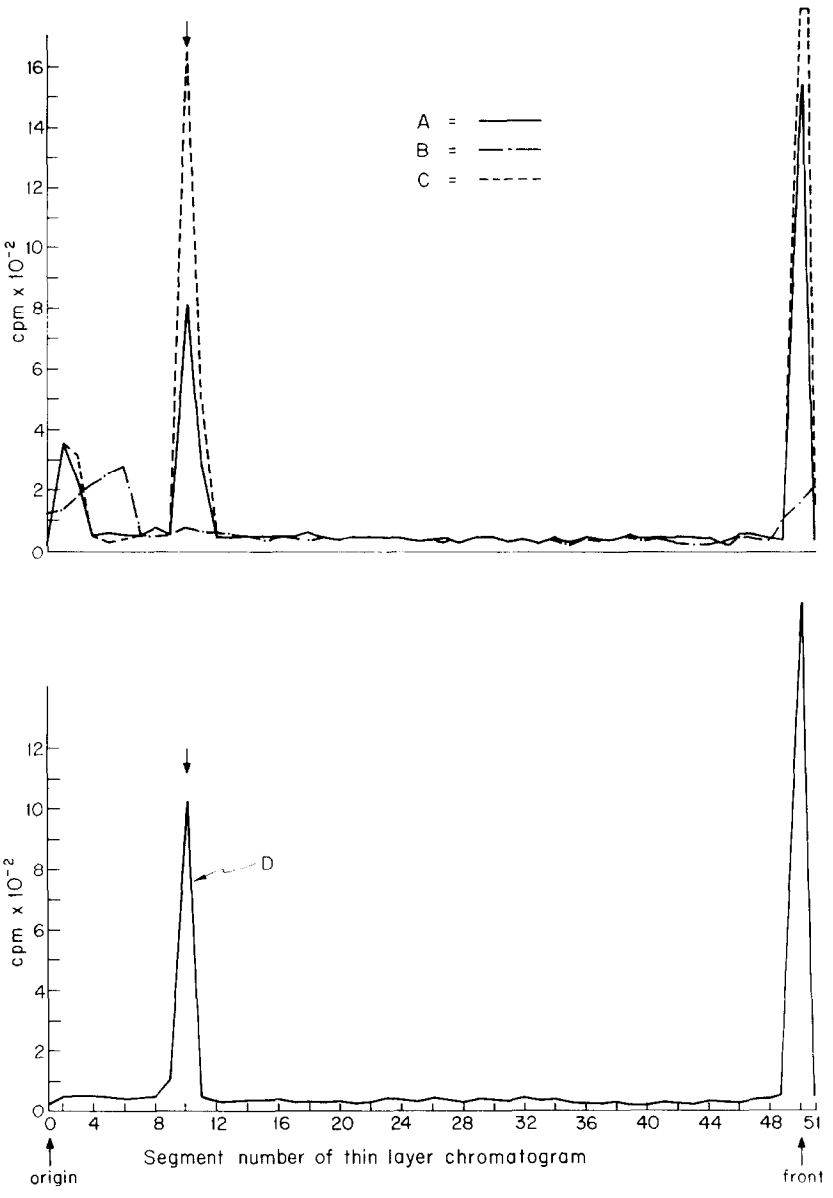

Fig. 2. Elution profile of the thin-layer chromatogram. Samples were assayed, extracted, and applied to TLC plates as described in Materials and Methods. Radioactivity was then measured in 2-mm segments. Key: (A) brain microvessels; (B) microvessels boiled in aqueous ammonia (tissue blank); (C) microvessels plus 1.5 pmoles of added serotonin; and (D) 1.5 pmoles of serotonin in aqueous media. The arrows at segment No. 9 mark the location of the authentic melatonin.

separates the isotopic melatonin formed from all other indoles in biological matter. Using this modification, 125 fmoles of serotonin or NAS can be detected at twice background levels of radioactivity. We used this method to measure the serotonin content of intraparenchymal microvessels from a single rat brain, and the serotonin and NAS levels from less than 15 per cent of a single rat pineal organ.

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Metabolism of digitoxin by isolated rat hepatocytes

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The metabolism of drugs in the liver can be studied at different levels of biological organization including the intact organ, cellular and subcellular levels. Studies at each organizational level will give useful information on types of reactions or kinetic parameters, but these also have their own limitations. In *In vivo* studies, complicating extrahepatic factors such as neurological, endocrinological and circulatory phenomena may influence the results. In studies using homogenates or microsomal preparations, the composition of the incubation medium is quite different from that of the cytosol of intact hepatocytes [1]. In addition, liver homogenates or microsomes are prepared from different cell populations constituting the liver. In view of these limitations, an experimental system based on the use of isolated hepatocytes has the advantage that extrahepatic influences can be excluded and that the isolated hepatocytes are intact cells. Therefore, the data obtained with isolated hepatocytes can be expected to provide useful information on the role of the liver in the kinetics of drug metabolism *in vivo*. In this paper, a study on the metabolism of the cardiac glycoside digitoxin (DT_3)* using isolated hepatocytes will be described.

Male 3-month-old Wistar rats (304 ± 26 g body wt., mean ± S.D.) were used. The animals were purchased when they were 11 weeks old (Shizuoka Jikkendobutsu, Shizuoka, Japan) and maintained on commercial rat pellets in the animal laboratory of the institute (four animals to a cage). Room temperature was kept constant (22 ± 2°). [^{3}H]DT_3 (10.9 Ci·mmole^{-1}, generally labeled) was purchased from New England Nuclear, Boston, MA., U.S.A.; DT_3 and digoxin (DG_3) from Merck, Darmstadt, F.R.G.; digitoxigenin-bis-digitoxoside (DT_2), digitoxigenin-mono-digitoxoside (DT_1), digitoxigenin (DT_0), digoxigenin-bis-digitoxoside (DG_2), digoxigenin-mono-digitoxoside (DG_1) and digoxigenin (DG_0) from Roth, Karlsruhe, F.R.G.; Amberlite XAD-2 resin from Rohm and Haas Nederland BV, Dordrecht, The Netherlands; the enzymes collagenase (type 1) and hyaluronidase (type 1) from Sigma, St. Louis, MO, U.S.A. and Waymouth MB 752/1 medium from GIBCO, Grand Island, NY, U.S.A.

Unlabelled and [^{3}H]DT_3 were added to a dimethyl sulfoxide (DMSO) solution. The final concentration of DMSO in the incubation medium was 0.5%. No damage to the isolated hepatocytes was to be expected at this concentration [2]. The degree of dissolution of DT_3 at this concentration was determined by means of ultracentrifugation and the millipore filter method. Thin-layer chromatography (t.l.c.) was performed with DC Fertigplatten Kieselgel 60 of 20 × 20 cm dimension (Merck, Darmstadt, F.R.G.). The hepatocytes were isolated by perfusion and incubation of the liver with the enzymes collagenase and hyaluronidase as reported earlier [3, 4]. The concentration of the cells was determined with a hemocytometer.

The method for determining DT_3 biotransformation was as follows. A known number of hepatocytes in 4 ml Waymouth MB 752/1 medium were incubated with various amounts of unlabelled DT_3 mixed with [^{3}H]DT_3. The pH of the medium was kept at 7.4, which was found to be optimal for digitoxin metabolism [5]. The incubation was carried out at 37° under an atmosphere of 95% O_2 and 5% CO_2 with constant shaking (100 oscillations·min^{-1}). Immediately after the addition of DT_3 and after various incubation periods, 0.5-ml samples of the cell suspension were withdrawn and added to 1.5 ml ethanol [6]. The mixture was centrifuged at 3000 *g* for 10 min [6]. The supernatant fraction was evaporated and then layered on a column packed with Amberlite XAD-2 resin using 6 ml water. The column was washed with 20 ml distilled water to remove the constituents of the incubation medium such as salts and sugars. DT_3 and its metabolites were eluted from the column with 20 ml ethanol. The ethanol fraction was evaporated *in vacuo* at 37°; the residue was dissolved in about 100 μl of chloroform:methanol, 1:1. The solution

* Abbreviations used: DT_3, digitoxin; DT_2, digitoxigenin-bis-digitoxoside; DT_1, digitoxigenin-mono-digitoxoside; DT_0, digitoxigenin; DG_3, digoxin; DG_2, digoxigenin-bis-digitoxoside; DG_1, digoxigenin-mono-digitoxoside; DG_0, digoxigenin; DMSO, dimethyl sulfoxide; t.l.c., thin-layer chromatography.