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Note

Simultaneous micro-determination of serotonin and 5-hydroxyindole-3-acetic acid with 5-hydroxy-N_{ω}-methyltryptamine, as an internal standard, in biological materials by high-performance liquid chromatography with electrochemical detection

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Serotonin (5-hydroxytryptamine; 5-HT), which is involved in many biological and physiological functions [1–3], is found not only in neurones, but also platelets, mast cells and the enterochromaffin cells [4]. Only ca. 1–2% of 5-HT in the whole body is detected in the brain and the residual 5-HT is in the peripheral tissues and fluids [4]. Although several papers have described the use of high-performance liquid chromatography (HPLC) to measure 5-HT, the most applicable methods have used samples of brain, cerebrospinal fluid or plasma [5–15]. However, since much 5-HT is known to be contained in whole blood, especially platelets [16], the estimation of 5-HT in whole blood would be preferable and would avoid the time-consuming isolation of platelets. However, the information about the HPLC analysis of 5-HT in whole blood is not always practical from the point of view of recovery and/or pretreatment [17,18]. During experiments to examine the best preparative method, we found that 5-HT and its related compounds were better prepared from whole blood with trichloroacetic acid than with perchloric acid [17,18].

Furthermore, it is also important to measure the urinary content of 5-hydroxyindole-3-acetic acid (5-HIAA), because urinary excretion of 5-HIAA increases in malignant carcinoid patients [19,20]. Urinary 5-HIAA has generally been

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measured by HPLC after Sephadex column chromatographic separation. However, this pretreatment is time-consuming and requires particular skills [20,21].

In this paper, we describe an HPLC method with electrochemical detection (ED) for the determination of 5-HT and 5-HIAA in the whole blood, urine and various tissues of the rat using 5-hydroxy-N_{ω}-methyltryptamine (methyl serotonin; M-5-HT) as an internal standard without a clean-up procedure. The method is highly sensitive, simple and practical.

EXPERIMENTAL

Chemicals

5-HIAA, M-5-HT oxalate and sodium 1-octanesulphonate (SOS) were obtained from Aldrich (Milwaukee, WI, U.S.A.). 5-HT-creatinine sulphate and L-cysteine monohydrochloride monohydrate were purchased from Wako (Osaka, Japan). Sodium 1-pentanesulphonate (SPS) was acquired from Nakarai (Kyoto, Japan). All other chemicals used were of the highest purity obtainable from commercial sources.

Liquid chromatography

The apparatus employed consisted of a liquid chromatograph P-530 (Irica Instruments, Kyoto, Japan), a 7-ODS-L column (250 mm×4.6 mm I.D., particle size 7 μm) (Chemco Scientific, Osaka, Japan) and an Irica E-502 amperometric detector with a glassy carbon electrode for analyses of blood and organ tissue samples. The mobile phase was a mixture of a degassed solution of 40 m*M* potassium dihydrogenphosphate containing 3 mg/l Na_2 EDTA and 100 mg/l SPS (pH 4.5)–methanol (9:1), v/v) and was used at a flow-rate of 1.0 ml/min. The applied voltage was set at +600 mV. When urine samples were analysed unknown peaks interfered with the identification of 5-HT, 5-HIAA and M-5-HT. For the analysis of urine samples, the following conditions were used: column, 5-ODS-H (150 mm×4.6 mm I.D., particle size 5 μm); mobile phase, 40 m*M* potassium dihydrogenphosphate containing 3 mg/l Na_2 EDTA and 100 mg/l SOS (pH adjusted to 3.5 by the addition of concentrated phosphoric acid)–methanol (8:2, v/v); flow-rate, 0.7 ml/min. Deionized, doubly glass-distilled water was used for chromatography. The HPLC system was interfaced with a Shimadzu Chromatopac C-R6A (Shimadzu, Kyoto, Japan) for data processing.

Animal experiments

Male rats of the Sprague–Dawley strain (six weeks old), obtained from Clea Japan, were fed a 20% casein diet (20% vitamin-free milk casein, 68.9% sucrose, 5% soybean oil, 5% Harper's mineral mixture and 1% Harper's vitamin mixture, 1500 I.U. of vitamin A per 100 g diet, 150 I.U. of vitamin D per 100 g diet and 0.6 mg of vitamin E per 100 g diet) for twelve days. The rats were kept individually in metabolic cages. Urine was collected for the last two days in a tinfoil-wrapped amber bottle containing 1 ml of 1 *M* hydrochloric acid and 1% cysteine. When the reference 5-HT (516 nmol) and 5-HIAA (297 nmol) were dissolved in 0.1 *M* hydrochloric acid containing 0.1% cysteine and placed in a tinfoil-wrapped amber

bottle kept in the animal room, they were stable at least for four days. The room temperature was 22 ± 2°C and the humidity ca. 60%. The light–darkness cycle was 6:00–18:00 (light) and 18:00–6:00 (darkness). The rats were killed by decapitation at ca. 9:00 a.m., and blood and organ tissues were removed as rapidly as possible.

Sample preparation

All procedures were performed at 4°C in the dark. Fresh organs (1 g) were homogenized in 5 ml of cold 0.6 *M* perchloric acid containing 0.1% cysteine and 1.07 μg/ml M-5-HT (volume of homogenate, 5.3 ml). The homogenate (50 μl) was diluted with 0.95 ml of cold 0.6 *M* perchloric acid containing 0.1% cysteine. The diluted mixture was mixed for 10 min, allowed to stand for 5 min and then centrifuged for 3 min at 10 000 *g*. The resulting supernatant (volume of the supernatant, 0.98 ml) was filtered through a 0.45-μm filter, and an aliquot (10 μl) was analysed directly by HPLC. Whole blood (10 μl) was treated with 0.99 ml of cold 5% trichloroacetic acid containing 0.1% cysteine and 0.06 μg/ml M-5-HT. Urine (50 μl) was treated with 0.95 ml of cold 0.6 *M* perchloric acid containing 0.1% cysteine and 1.07 μg/ml M-5-HT. The procedures for blood and urine were same as for organs. The volumes of supernatants of blood and urine were 0.99 and 1.00 ml, respectively. An aliquot (10 μl) of blood sample and an aliquot (5 μl) of urine sample were analysed directly by HPLC.

M-5-HT was used as an internal standard in the analyses of all biological samples. Samples were analysed within three days after they were prepared. Standard 5-HT (1 nmol/ml in 0.6 *M* perchloric acid containing 0.1% cysteine), 5-HIAA (1 nmol/ml) and M-5-HT (1 nmol/ml) were stable at least for five days at 4°C in the dark.

RESULTS AND DISCUSSION

Separation of 5-HT, 5-HIAA and M-5-HT

The retention of the amines (5-HT and M-5-HT) increases with increasing length of the hydrophobic chain of the ion-pair reagent (SOS versus SPS). 5-HIAA elutes faster at a higher pH (22.98 min at pH 3.5 and 14.88 min at pH 4.5). A typical chromatogram of the reference 5-HT, 5-HIAA and M-5-HT obtained under the conditions described for the analyses of blood and organ tissue samples is shown in Fig. 1A. Fig. 2A shows a typical chromatogram of reference 5-HT, 5-HIAA and M-5-HT under the conditions used for analysis of urine samples. The total analysis time was ca. 18 min in both cases.

Linear range and limit of detection

The concentrations of 5-HT, 5-HIAA and M-5-HT in 0.6 *M* perchloric acid containing 0.1% cysteine were calculated using values for the molar absorptivity of 5500, 5600 and 5600 at 275 nm, respectively. The calibration curves for 5-HT and 5-HIAA were linear in the range from 50 fmol to 100 pmol per injection, with correlation coefficients of 0.999. The integrated peak areas of 5-HT and 5-HIAA were 83 454 ± 1973, 79 020 ± 1751 per pmol (mean ± S.D., nine different concen-

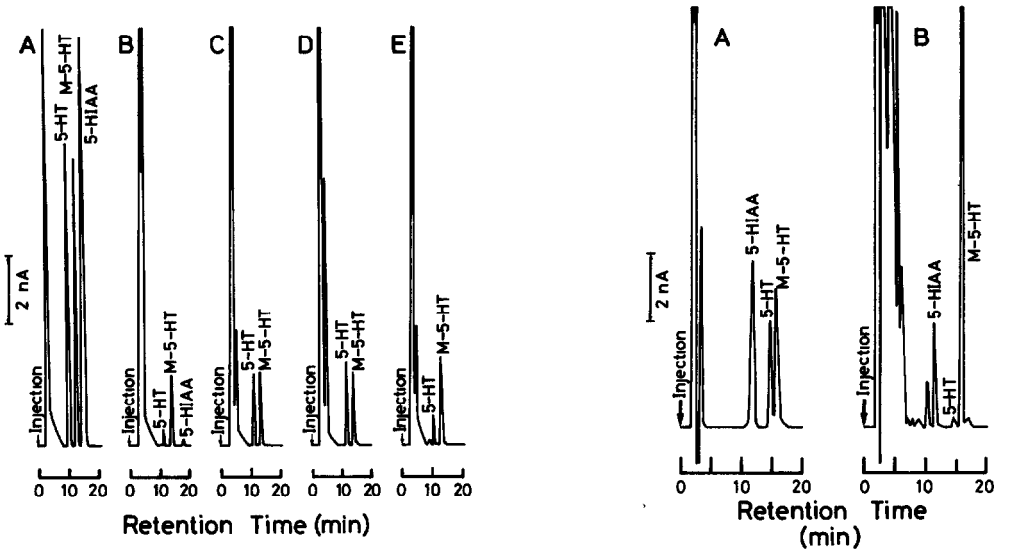

Fig. 1. (A) Chromatogram of standard 5-HT (6.36 pmol), M-5-HT (7.08 pmol) and 5-HIAA (12.27 pmol). (B) Chromatogram of the extract of rat brain: sample size, 10 μl containing 0.32 pmol of 5-HT, 1.80 pmol of M-5-HT and 0.09 pmol of 5-HIAA. (C) Chromatogram of the extract of rat stomach: sample size, 10 μl containing 1.53 pmol of 5-HT and 1.78 pmol of M-5-HT. (D) Chromatogram of the extract of rat small intestine: sample size, 10 μl containing 1.73 pmol of 5-HT and 1.79 pmol of M-5-HT. (E) Chromatogram of the extract of rat blood: sample size, 10 μl containing 0.61 pmol of 5-HT and 2.11 pmol of M-5-HT. For conditions, see *Liquid chromatography*.

Fig. 2. (A) Chromatogram of standard 5-HT (1.51 pmol), 5-HIAA (1.96 pmol) and M-5-HT (2.36 pmol). (B) Chromatogram of the extract of rat urine: sample size, 5 μl containing 1.20 pmol of 5-HIAA, 0.13 pmol of 5-HT and 18.15 pmol of M-5-HT. For conditions, see *Liquid chromatography*.

trations used), respectively. The amounts of 5-HT and 5-HIAA were calculated by the following equations: 5-HT (pmol) = integrated peak area/83 000 and 5-HIAA (pmol) = integrated peak area/79 000, respectively. The detection limits of 5-HT and 5-HIAA were 50 fmol (8.81 pg) and 50 fmol (9.56 pg), respectively, at a signal-to-noise ratio of 5:1.

Recoveries of 5-HT, 5-HIAA and M-5-HT

A 5-μl volume of 40 nmol/ml 5-HT (200 nmol) and 5 μl of 40 nmol/ml 5-HIAA (200 nmol) were added to 50 μl of the organ tissue homogenate (190 pmol of M-5-HT already contained) and was prepared as described in *Sample preparation* (in this case, the volume of the supernatant was 990 μl). Then 5 μl of 20 nmol/ml 5-HT (100 pmol) and 5 μl of 20 nmol/ml 5-HIAA (100 pmol) were added to the mixture of 990 μl of cold 5% trichloroacetic acid, containing 0.1% cysteine and 0.06 μg/ml M-5-HT, and 10 μl of blood. The blood sample was prepared as described in *Sample preparation* (in this case, the volume of the supernatant was 1000 μl). Then 5 μl of 100 nmol/ml 5-HT (500 pmol) and 5 μl of 100 nmol/ml of 5-HIAA (500 pmol) were added to the mixture of 950 μl of cold 0.6 *M* perchloric acid, containing 0.1% cysteine and 1.07 μg/ml M-5-HT, and 50 μl of urine. The

TABLE I

RECOVERIES OF 5-HT, 5-HIAA AND M-5-HT (INTERNAL STANDARD) FROM BIOLOGICAL MATERIALS

For detailed experimental conditions for 5-HT and preparation of the related compounds, see text.

Sample	Recovery (mean ± S.D., $n=4$) (%)		
	5-HT	5-HIAA	M-5-HT
Brain[a,b]	95.8 ± 6.2	92.4 ± 7.4	92.4 ± 7.8
Stomach[a,b]	93.3 ± 6.0	85.4 ± 4.6	87.9 ± 3.4
Small intestine[a,b] (upper part 30 cm)	96.5 ± 6.4	95.7 ± 5.8	90.4 ± 3.4
Blood[c,d]	88.3 ± 4.2	93.9 ± 3.8	86.1 ± 2.6
Urine[a,e]	101.3 ± 2.6	98.8 ± 1.2	99.7 ± 2.0

[a]Perchloric acid preparation.
[b]Recovery (%) = (integrated peak area of endogenous 5-HT or 5-HIAA + added 5-HT, 5-HIAA or M-5-HT per 10 μl) − (integrated peak area of endogenous 5-HT or 5-HIAA per 10 μl) × (990 μl/10 μl) × (1/integrated peak area of added 5-HT, 5-HIAA or M-5-HT) × 100.
[c]Trichloroacetic acid preparation.
[d]Recovery (%) = (integrated peak area of endogenous 5-HT or 5-HIAA + added 5-HT, 5-HIAA or M-5-HT per 10 μl) − (integrated peak area of endogenous 5-HT or 5-HIAA per 10 μl) × (1000 μl/10 μl) × (1/integrated peak area of added 5-HT, 5-HIAA or M-5-HT) × 100.
[e]Recovery (%) = (integrated peak area of endogenous 5-HT or 5-HIAA + added 5-HT, 5-HIAA or M-5-HT per 5 μl) − (integrated peak area of endogenous 5-HT or 5-HIAA per 5 μl) × (1010 μl/5 μl) × (1/integrated peak area of added 5-HT, 5-HIAA or M-5-HT) × 100.

urine sample was prepared as described in *Sample preparation* (in this case, the volume of the supernatant was 1010 μl). The recoveries of 5-HT, 5-HIAA and M-5-HT from organ tissues were similar (Table I) to those previously reported [10,11]. The recoveries of 5-HT, 5-HIAA and M-5-HT from whole blood were each ca. 90% (Table I). The recoveries of the present method with 5% trichloroacetic acid containing 0.1% cysteine (each of final concentration) were much better than those obtained either with 0.49 *M* perchloric acid containing 0.2 *M* ascorbic acid (each of final concentration) (recovery 59 ± 5%) [17] or by bubbling carbon monoxide through the whole blood sample before precipitation proteins with 0.4 *M* perchloric acid (final concentration) (recovery 61 ± 4%) [18]. The recoveries of 5-HT, 5-HIAA and M-5-HT from urine were each ca. 100% (Table I). Fornstedt [20] and Iinuma et al. [21] also obtained 100% recoveries of 5-HIAA from urine by using a Sephadex column. Narasimhachari and Landa [12] reported an HPLC method for 5-HT in urine after ethyl acetate extraction, for which the recovery of 5-HT from urine was not reported. Compared with their methods, our preparative method is more straightforward.

5-HT and 5-HIAA contents in rat brain, stomach, small intestine, blood and urine

Chromatograms of samples of rat brain, stomach, small intestine and whole blood are shown in Fig. 1B–E and a chromatogram of a sample of rat urine is shown in Fig. 2B. 5-HT and 5-HIAA in biological materials were characterized

TABLE II

LEVELS OF 5-HT AND 5-HIAA IN BRAIN, STOMACH, SMALL INTESTINE, BLOOD AND URINE

Values are mean ± S.D. for five rats; N.D. = not determined.

Sample	5-HT	5-HIAA
Brain (nmol/g)	2.14±0.18	1.25± 0.11
Stomach (nmol/g)	13.72±1.63	N.D.
Small intestine (upper part 30 cm) (nmol/g)	26.38±3.53	N.D.
Blood (nmol/ml)	5.43±0.31	N.D.
Urine (nmol/day)	7.18±5.72	144.54±22.25

on the basis of their retention times. The contents of 5-HT and 5-HIAA are given in Table II.

The electrochemical detector is much more sensitive than the fluorimeter or other commonly used detection devices for hydroxyindoles [5]. It is important to emphasize that perturbation of either the plexiglass housing or the electrode surface will cause turbulent flow over the electrode and thus reduce the limit of detection [22]. However, since the electrochemical detector used (Irica E-502 amperometric detector) is highly sensitive and stable for at least three months of uninterrupted performance, 5-HT and the related compounds in small amounts of biological extracts can be analysed on a routine basis. We believe that this method will be practical and useful in the field of 5-HT research.

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