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Note

High-performance liquid chromatographic determination of 5-hydroxyindole-3-acetic acid in urine using Sephadex G-10 for isolation

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It is well known that certain carcinoid tumors may be characterized by a release of serotonin (5-hydroxytryptamine, 5-HT), and that urine from patients with metastatic carcinoids contains abnormally high amounts of 5-hydroxyindole-3-acetic acid (5-HIAA), the main metabolite of 5-HT. Some of the methods to measure 5-HIAA in urine have been discussed by the author [1] who also presented a simple, sensitive and selective two-step chromatographic procedure. In that work, dinitrophenyl-coupled, thiolated Sephadex G-25 (DNP-S-Seph.G-25) was synthesized and used for the chromatographic isolation in combination with quantitative analysis by reversed-phase high-performance liquid chromatography (HPLC), using UV-absorbance detection. The present paper reports a further simplification utilizing Sephadex G-10 instead of the synthesized adsorbent. The new method was used to investigate the 5-HIAA excretion in urine from patients with carcinoid syndrome as well as from healthy individuals.

EXPERIMENTAL

Apparatus

The chromatographic isolation equipment [1] was modified as follows. The pump was replaced by a 13-canal micro-pump, MP 13 A (Ismatec, Zurich, Switzerland), with Technicon SMA flow rated pump tubes, flow-rate 0.05 ml/min (Technicon, Tarrytown, N.Y., U.S.A.). As isolation columns (4.5 cm × 4 mm I.D.) pipettes were utilized as before but were cut off leaving 12 cm and marked at a height of 4.5 cm. For a separate study (Table I), 30 cm × 4 mm I.D. columns were made from intact pipettes, marked at a height of 30 cm. Except for this study the fraction collector was omitted. The previously described [1] HPLC—UV equipment was used without any modification.

Reagents

See ref. 1, Reagents, for (1) buffer, (2) mobile phase and (3) a stock solution of 5-HIAA (100 μg/ml) in the buffer, checked and stored as described (diluting to 25 μg/ml resulted in A_{280} = 0.710 S.D. ± 0.015, n = 5, for each of the two lots); prepare the working standards 25, 12.5 and 6.25 μg/ml by serial dilution from stock solution as previously described; (4) pack the 4.5 cm × 4 mm I.D. columns with Sephadex G-10 (Pharmacia, Uppsala, Sweden) and equilibrate with about 20 ml buffer as in ref. 1, Preparation of isolation columns. Store the packed columns tightly capped at 5°, without any preservative in the buffer; (5) pack as described in (4) the 30 cm × 4 mm I.D. columns with the respective gel Sephadex G-10, G-15, G-25 and LH-20 (Pharmacia), Biogel P-2 (Bio-Rad Labs., Richmond, Calif., U.S.A.), and DNP-S-Seph.G-25 used in the earlier [1] work (for a modified synthesis, see pp. 54—55 in ref. 2), and then equilibrate each packing with about 40 ml of buffer (store the 30-cm columns as described for the 4.5-cm ones); (6) other reagents: blue silica gel (Grace, Homburg, G.F.R.), 4-hydroxy-3-methoxymandelic acid (VMA), 4-hydroxy-3-methoxyphenylacetic acid (HVA), tryptophan (Trp), tryptamine (T) and indole-3-acetic acid (IAA) (Sigma, St. Louis, Mo., U.S.A.).

Procedure

Calibration process. Place a set of one to three columns, 4.5 cm × 4 mm I.D., packed with Sephadex G-10 and equilibrated, over one to three sets of 15 small test tubes, marked for 1.0 ml. Suck off the buffer above the bed with a disposable Pasteur pipette, add 500 μl of a 25 μg/ml standard solution with a capillary pipette and allow to drain completely into the gel. Wash by means of 2 or 3 drops of the buffer and allow to drain. Refill the empty space with the buffer, connect the columns with the pump (flow-rate 3 ml/h), and collect 1-ml fractions. Monitor the absorbance of the individual fraction at 280 nm. Inject solution from each of the found UV-positive fractions, Nos. 7—12, and from both fractions beyond, Nos. 6 and 13, onto the reversed-phase column (PXS 1025, Partisil-10 ODS). Note the presence of 5-HIAA on the basis of the retention time by comparison with a directly injected standard, and establish the range of positive fractions.

Sample preparation. Ensure non-intake of banana or pineapple within 24 h before and during any urine collecting [3]. Collect and store 24-h urine specimens as described in ref. 1. For morning urine (voided after fasting overnight), note the volume, keep 9.7 ml and mix with 0.3 ml of glacial acetic acid, filter, and store as 24-h specimens; analyze at once or within 2 weeks.

Determination of 5-HIAA in urine. Carry out the determination as described for the calibration above with following modifications. Place a set of one to ten calibrated 4.5 cm × 4 mm I.D. columns over one to ten sets of two 15-ml Präzision test tubes (Scherf, Ostheim, G.F.R.), graduated for 10 ml. Run the samples (500 μl) on tinfoil-wrapped isolation columns. Collect 6 ml and discard. Collect 7 ml in the second test tube (tinfoil-wrapped), and keep for separation and quantitative determination on the HPLC—UV system. Prior to injection, check the system by direct injection of a 5-HIAA standard onto the ODS column, conditioned with the mobile-phase eluent (average peak height for a 25 μg/ml standard at 0.16 a.u.f.s. is 92 ± 2 mm). Obtain the 5-

HIAA concentration via a calibration curve (peak height in mm versus concentration in μg/ml), constructed by means of the standard solutions 25, 12.5 and 6.25 μg/ml, run in the same way as the samples. Concentrations greater than 25 μg/ml will require dilution of the urine samples. For quantities less than 1 μg/ml, concentrate as follows: pour 2 ml of the second fraction (7 ml) into a test tube (4 cm × 15 mm I.D.), placed in a desiccator, evaporate in vacuum at room temperature in the presence of blue silica gel, dissolve the residue quantitatively by washing the tube walls with 0.4 ml of the buffer, and inject at once onto the ODS column.

After using, regenerate the G-10 columns by eluting with about 20 ml buffer. Flush the ODS column daily with methanol, and store in methanol at 5°.

RESULTS AND DISCUSSION

A study on isolation ability of some available gels

To ascertain whether DNP-S-Seph.G-25 [1] could be replaced with a commercially available gel, a comparison between 30 cm × 4 mm I.D. columns packed with various gels (see Reagents) was performed. The elution positions of 5-HIAA were established in the same chromatographic conditions (Table I). By using Sephadex G-10, a threefold retention was attained; the retention was also found to be greater on G-15 (about 50%), while no difference appeared on LH-20. A comparison of the elution position of 5-HIAA with a screening of UV-positive urine compounds on G-10 (Fig. 1) showed that G-10 is the gel of choice for isolation of 5-HIAA from urine.

In order to obtain additional information on the isolation ability of the above packings, the retention behaviour of the aromatic urine metabolites VMA, HVA, Trp, T, 5-HT and IAA was studied. Table I shows how the reten-

TABLE I

RETENTION OF SOME AROMATIC COMPOUNDS, RUN ON SOME UNCHARGED HYDROPHILIC* GELS

Eluted compound	Elution range on respective gel, as Nos. of UV-positive fractions**					
	G-10	G-15	G-25	LH-20	Biogel P-2	DNP-S-Seph.G-25
VMA	6—10	5—6	4—5	4—6	4—5	5—6
HVA	10—14	7—9	7—8	8—10	4—5	8—9
Trp	7—9	6—8	3—5	4—5	4—5	7—9
T	6—10	5—7	3—6	3—4	4—5	8—10
5-HT	9—13	8—11	3—6	5—6	4—5	11—15
IAA	44—56***		5—8	15—22		18—22
5-HIAA	52—64***	21—29	7—10	18—23	7—10	18—24

*LH-20 and DNP-S-Seph.G-25 are also partly hydrophobic.

**250 μl of a 40 μg/ml solution of respective compound (IAA concentration unknown) chromatographed on a 30 cm × 4 mm I.D. column in 0.1 *M* ammonium formate buffer, pH 3 (flow-rate 3 ml/h). Fractions of 1 ml collected and monitored at 280 nm.

***If run in 0.1 *M* ammonium formate buffer, pH 8, IAA was eluted in Nos. 5—7 and 5-HIAA in Nos. 7—9.

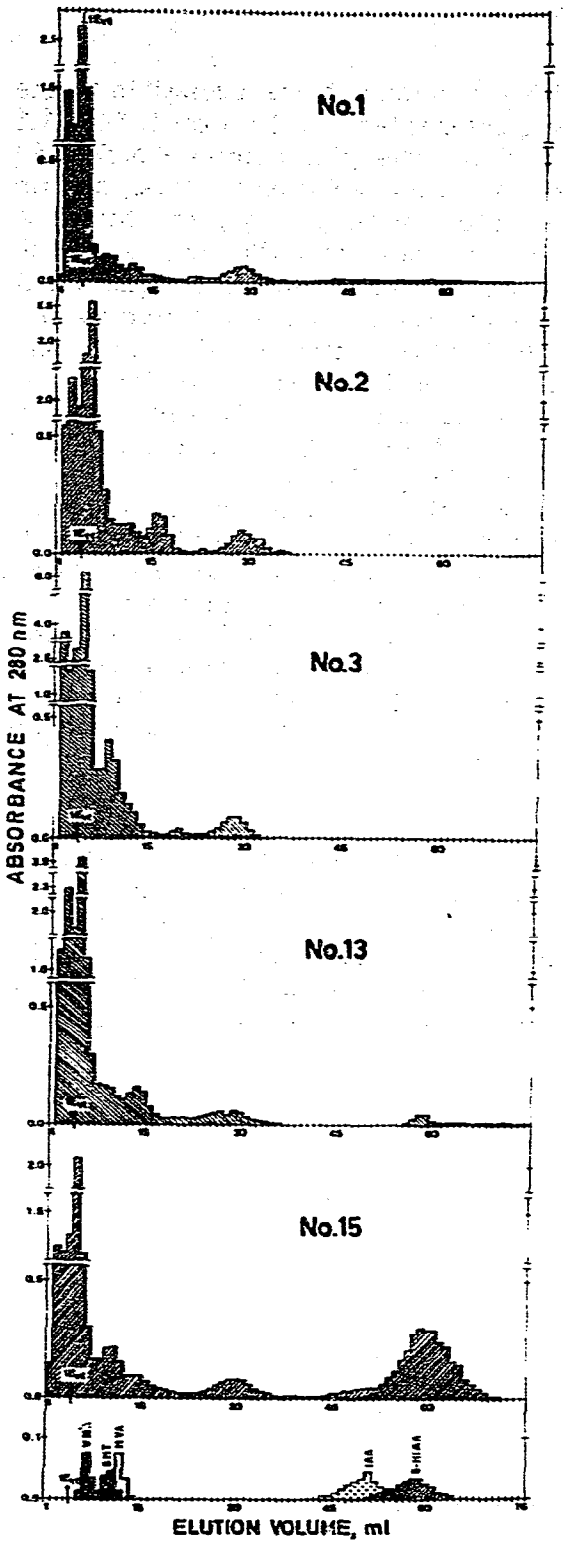

Fig. 1. Sephadex G-10 chromatograms of some aromatic compounds (bottom) and of 24-h urine specimens from carcinoid patients (subject Nos. 13 and 15) and from healthy individuals (subject Nos. 1—3). Conditions as in Table I, second footnote.

tion on the investigated G-gels varies with the amount of gel accessible to the solute: the tighter the cross-linking, the greater retention. Run on G-10, IAA was retarded to the same magnitude as 5-HIAA. The other compounds showed moderate adsorption as reported for aromatic amino acids on G-10 [4] and on G-25 [5]. The occurrence of a ring-substituted hydroxyl group was found to enhance adsorption sufficiently to allow separation of 5-HT from T and 5-HIAA from IAA with little overlapping. On the other hand, the replacement of a hydrogen on the α-carbon atom by a hydroxyl group was found to produce the opposite effect, since VMA, which elutes faster, could be fully separated from HVA. The elution behaviour on Biogel P-2 proved similar to that on Sephadex G-25 for most of the investigated compounds. However, a comparison with G-10 or G-15 could not be carried through, owing to the lack of gels with a tighter cross-linking than P-2 in the Biogel P series.

The retardation of 5-HIAA and IAA at pH 3 on G-10 was substantially reduced at pH 8 (Table I) and was almost doubled with 1 *M* sodium chloride in the buffer (unpublished results), pointing to hydrophobic interactions.

From the study one may conclude that Sephadex G-10 is the most suitable for isolation of the metabolites 5-HIAA, IAA, HVA and 5-HT. The isolation of metabolites from urine, such as Trp, T and VMA, which are least retarded on G-10, probably require cross-linked gels still tighter than G-10. Alternatively, G-10 covalently bounded to hydrophobic ligands, such as aliphatic chains or as aromatics containing more than one ring, may also be suitable.

5-HIAA analysis

The present method offers advantages over the previous one [1]. Besides eliminating the DNP-S-Seph.G-25 synthesis, an enhanced sensitivity is attained (Fig. 2) since the stronger adsorption allows use of shorter columns with less total bed volume (0.56 ml) and application of a double sample volume (0.50 ml). The specificity [1] is secured still more on G-10, as the isolation of 5-HIAA from the other UV-positive endogenous urine metabolites (Fig. 1) is

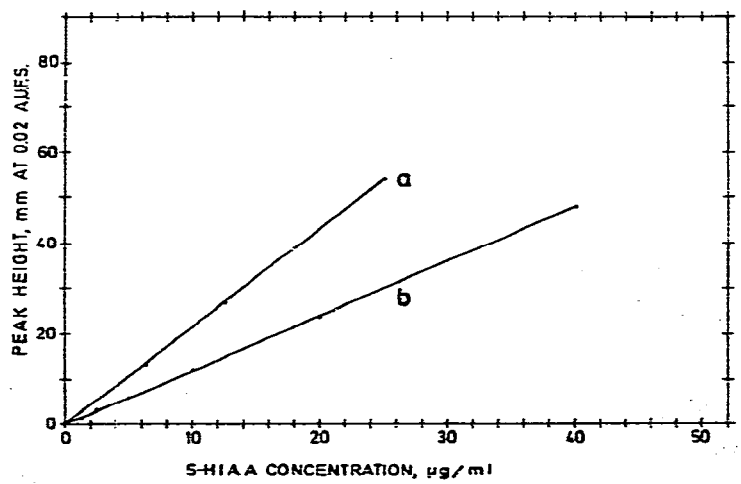

Fig. 2. 5-HIAA calibration curve of (a) the new and (b) the previous method.

TABLE II

DETERMINATION OF 5-HIAA IN URINE COLLECTED FROM HEALTHY INDIVIDUALS AND FROM PATIENTS WITH CARCINOID SYNDROME

No.*	Sex	Age	Weight (kg)	Urine (ml/24 h)	5-HIAA concentration in urine**			
					Previous method [1]		Present method	
					μg/ml	mg/24 h	μg/ml	mg/24 h
1	female	50	55	1440	3.3	4.6	3.3	4.6
2	male	62	79	1100	4.6	4.9	4.7	5.0
				1050	4.4	4.5	4.2	4.3
				690	6.7	4.5	6.6	4.4
3	female	18	63	550	7.0	3.7	7.2	3.8
6***	female	56	79	780	7.1	5.4	6.9	5.2
9	male	46	110	2060	2.8	5.6	2.8	5.6
16	female	55	67	1230			3.4	4.0
				300 §			2.3	
17	female	58	70	975			2.6	2.4
18	male	63	67	1375			3.8	5.1
19***	female	23	60	1070			4.0	4.2
20***	female	55	65	1320			3.8	4.9
12	female	62		1060			40.0	41.1
15	male	60		1340	439.6	571.5	446.4	580.3
				350 §			315.4	
21	male	56		500 §			24.7	

* Numero series started in ref. 1; from former subjects new samples collected at later occasions; No. 16, first new subject; No. 12, 15 and 21, patients under medical treatment.

** Corrected for acetic acid.

*** Took daily medicine containing levothyroxinnatrium.

§ Morning urine.

improved. The results, obtained by analyzing the same samples according to both methods, were almost identical (Table II). Fig. 3 illustrates typical HPLC chromatograms obtained by the new method. Table II shows the normal urinary 5-HIAA level, ranging from 2.4 to 5.6 mg per 24 h, found in specimens from ten healthy subjects, as well as the values in urine from some carcinoid

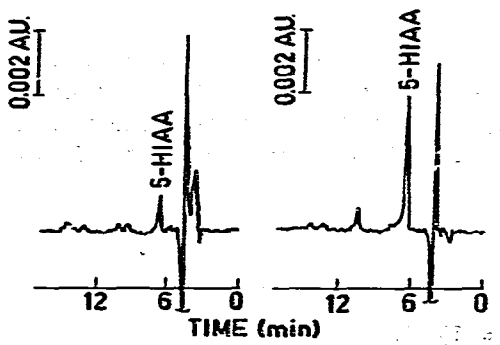

Fig. 3. Typical HPLC chromatograms of 5-HIAA in urine, obtained by the present method. A 24-h urine specimen from the healthy subject No. 2, containing 6.6 μg/ml (left), and a morning urine specimen from the carcinoid patient No. 21, containing 24.7 μg/ml (right).

TABLE III

TYPICAL ELUTION PROFILES OF 5-HIAA ON SEPHADEX G-10 COLUMNS OF DIFFERENT HEIGHT

500 µl of a 25 µg/ml standard solution chromatographed as in Calibration process, but injections onto ODS from each 1-ml fraction; operating as in Apparatus.

Column dimensions (cm × mm I.D.)	G-10, lot No.	Peak height (mm), measured in 1-ml fractions														
		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
4.5 × 4	2042	0	0	0	0	0	0	16	39	94	96	66	34	6	0	0
5.0 × 4	2042	0	0	0	0	0	0	0	3	17	67	115	100	40	15	3
4.5 × 4	6789	0	0	0	0	0	0	15	43	86	105	75	33	7	0	0

patients. The recovery of added 5-HIAA (6.25, 12.5 and 25 µg, respectively, per ml urine) was found to be 99.9% (R.S.D. = 0.5%; $n = 3 \times 5$). For quantitative performance on the small G-10 columns it is necessary to maintain the calibrated elution range intact: any drying phenomenon or any air bubble, appearing on storing, necessitates repacking and recalibration. Concerning columns of this small size, a few mm divergence in bed height causes a range displacement of one or two ml (Table III), followed by 5-HIAA losses of up to 5% and should be avoided by checking the height prior to the start and by readjusting.

Some of the patient specimens contained only low amounts of 5-HIAA, even if significantly exceeding the upper limit of the normal level, but the samples in question were collected after medical treatment (see also ref. 1). In general, patients with carcinoid syndrome were found to excrete extremely high amounts of this metabolite (ref. 1 and refs. 6—8 therein). However, such patient specimens, when available for analysis, are commonly collected from subjects taken for medical investigation in already advanced stages of the disease. Thus the possibility of detecting the tumor in its early stages calls for chemical detection in connection with healthy controls of groups in risk zones. The presented method may provide a suitable tool for these purposes as well.

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