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QUANTITATION OF TRYPTOPHAN METABOLITES IN RAT FECES BY THIN-LAYER CHROMATOGRAPHY

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SUMMARY

Indole, skatole, indole-3-acetic acid, indole-3-propionic acid, tryptamine, and free tryptophan have been extracted from rat fecal samples and quantitated by thin-layer chromatography. Additional tryptophan metabolites have been recovered in yields of 87–97% from supplemented fecal samples. Detection limits for all the compounds studied were in the 0.1 μg/g feces range. Quantitation was possible in the μg/g range with an estimated accuracy of ± 10%.

INTRODUCTION

Interest in the possible role of tryptophan metabolites in the etiology of colon cancer has prompted the development of a method for quantitating several of the metabolites in feces. Although many procedures have been used for analyzing tryptophan metabolites in urine[1–13], the existing methods for determining indoles[14–21] and amino acids[21–25] in feces were either laborious, relatively insensitive, or not applicable to rat feces. We have developed a method employing an initial ethanol homogenization of feces followed by liquid–liquid extraction. Subsequent analysis by thin-layer chromatography (TLC) permits quantitation in the μg/g range of acid, neutral and basic indoles, and certain amphoteric metabolites of tryptophan.

EXPERIMENTAL

Materials

Ethanol (95%) was purchased from U.S. Ind. Chem. (New York, N.Y., U.S.A.). All other solvents used were spectroanalyzed grade as supplied by Fisher Scientific (Pittsburgh, Pa., U.S.A.). The diethyl ether was shaken with acidic ferrous sulfate before use. Tryptophan metabolite standards were purchased from Sigma (St. Louis, Mo., U.S.A.) and Aldrich (Milwaukee, Wisc., U.S.A.). Amberlite XAD-2 resin, supplied by Mallinckrodt (St. Louis, Mo., U.S.A.), was washed in bulk with

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methanol (2 × 2 volumes), then with distilled water (4 × 5 volumes) before use. Quantum Q-6 silica gel plates (20 × 20 cm) were purchased from A. H. Thomas (Philadelphia, Pa., U.S.A.).

*Extraction procedure**

Approximately 3.0 g of rat feces were collected fresh and placed in a tared weighing jar containing 15 ml of 95% ethanol. The contents were transferred to an homogenizing flask and 45 ml of 95% ethanol added. Homogenization was effected at high speed for 20 min. The homogenate was centrifuged for 20 min at 3250 *g* and the supernatant fluid decanted into a 250-ml round-bottom flask. The sample was brought almost to dryness on a rotary evaporator at 37° and 40 mm Hg. The flask was swirled with 40 ml of diethyl ether, then with 20 ml of dilute phosphoric acid (pH 2) and the solutions transferred to a separatory funnel. After shaking, the layers were separated and the aqueous layer was re-extracted with 40 ml of ether. The ether layers were combined and washed first with 20 ml, then 10 ml of phosphoric acid (pH 2). The combined aqueous fraction (A) was saved after having been washed with 20 ml of ether. The combined ether fraction (approx. 100 ml) was extracted twice with 30-ml portions of saturated aqueous sodium bicarbonate, and the bicarbonate fraction (B) saved after having been washed with 15 ml of ether. Forty milliliters of distilled water were added to the combined ether extract and the ether removed under a stream of nitrogen at 45°. The remaining warmed aqueous layer was filtered through a medium sintered glass filter, and the flask and filter rinsed with 10 ml of distilled water (45°). The filtrate was adjusted to pH 4 with 6 *M* phosphoric acid and extracted twice with 40 ml of ether. The ether was removed under a stream of nitrogen at 22° and the residue assayed by TLC (fraction I). The bicarbonate extract (B) was adjusted to pH 2 with concentrated phosphoric acid, and extracted three times with 30 ml of ether. The combined ether extract was washed with 15 ml of phosphoric acid (pH 2) and the ether removed under a stream of nitrogen at 35°. After careful transfer of the residue to a small test tube by four 2-ml rinses of ether, and removal of the ether, the sample was methylated for 15 min at 22° with 2 ml of etherial diazomethane, containing 10% methanol. The solution was then dried under nitrogen and the residue assayed by TLC (fraction II). The pH 2 aqueous fraction (A) was adjusted to pH 10.5 with concentrated ammonium hydroxide. The aqueous fraction (A′) was again saved after being extracted twice with 40-ml portions of ether. The combined ether extracts were dried under nitrogen and the residue assayed by TLC (fraction III). The aqueous fraction (A′) was adjusted to pH 6.0 with 6 *M* phosphoric acid and the trace of ether removed at 40° and 40 mm Hg on a rotary evaporator (approx. 15 min). Half (approx. 25 ml) of the solution was then chromatographed on a 1 × 18 cm column of Amberlite XAD-2 resin, which had been washed with 50 ml of distilled water. After a 10-ml wash of distilled water and aspiration of the column to remove traces of water, the column was eluted with 15 ml of methanol. The methanol fraction was dried on a rotary evaporator at 40° and 40 mm Hg. The residue was assayed by TLC (fraction IV).

*Thin-layer chromatography***

Fraction I was brought up in 200 μl of methanol and aliquots of 1–50 μl were

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* See Fig. 1 for outline.

** For abbreviations of compounds, see Table I.

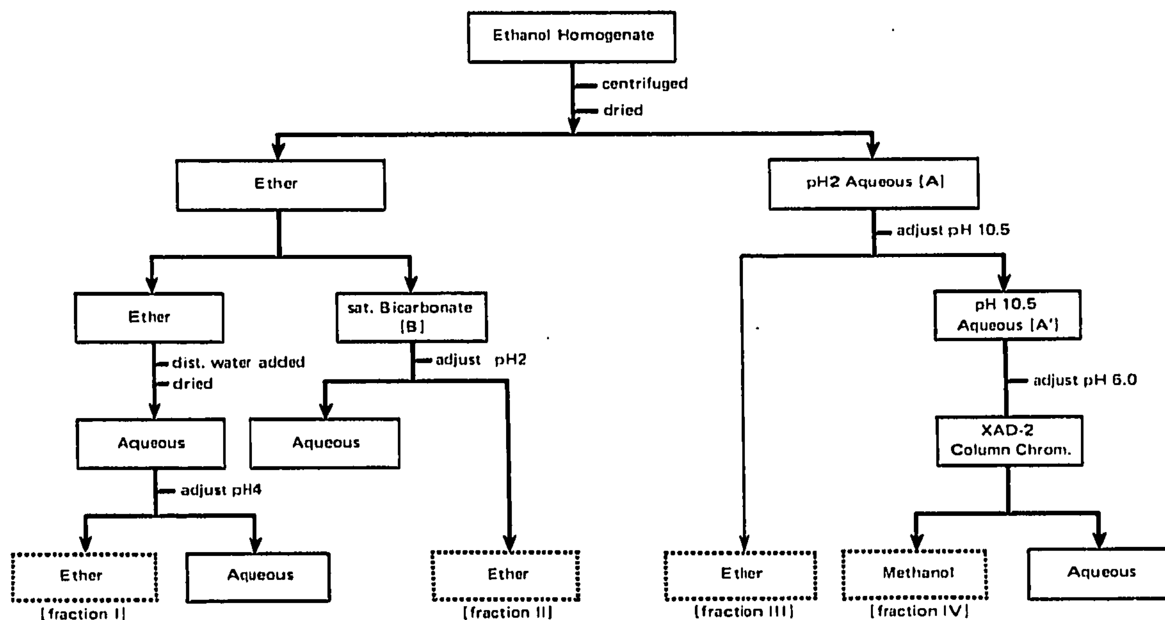

Fig. 1. Scheme for the extraction of tryptophan metabolites from rat fecal samples. See text for details.

TABLE I

$R_F \times 100$ VALUES AND COLOR REACTIONS OF METABOLITES

Solvent systems for TLC: A, Acetone–2-propanol–water–ammonia (0.88) (50:40:7:3) (ref. 26); B, trichloromethane–acetic acid–methanol–water (65:20:10:5) (ref. 26); C, benzene–acetone (90:10) (ref. 27). For the color reaction Ehrlich's spray reagent[28] (0.5 g *p*-dimethylaminobenzaldehyde–5 ml concentrated HCl–20 ml acetone) was used. The detection limits for the listed compounds with Ehrlich's reagent were approx. 0.05 μg after development.

Compound	*R_F value*			*Color reaction*
	A	*B*	*C*	
Indole (IND)	90	99	71	pink-maroon
Skatole (SKAT)	92	99	77	blue
Tryptophol (TOL)	90	97	18	grey-brown
Indole-3-acetic acid (IAA)	42	88	53*	blue
Indole-3-propionic acid (IPA)	57	97	60*	green-blue
Indole-3-lactic acid (ILA)	59	52	19*	gray-green
Indole-3-acrylic acid (IAyA)	48	93	38*	orange-brown
Anthranilic acid (ANTH)	52	96	79*	yellow
Indole-3-acetamide (IAMD)	88	95	2	blue
Tryptamine (TAM)	75	60	0	green-blue
Kynurenine (KYN)	25	31	0	orange
Tryptophan (TRP)	22	36	0	blue

* Methyl esters.

spotted on a Quantum Q-6 silica gel plate which had been activated for 30 min at 100°. A standard solution containing IND, SKAT, and TOL was spotted to give a range from 0.05 to 2.0 μg. The plate was run in solvent C (Table I), equilibrated beforehand for 30 min at 22°. After the solvent front had ascended 12 cm, the plate was sprayed evenly with 12 ml of Ehrlich's reagent (Table I). The plate was then heated at 100° for 2–3 min. Quantitation was accomplished by visual comparison of the spots in the sample having the same R_F values and color reactions as the standards. Fraction II was chromatographed in the same manner as fraction I with a standard solution of the methyl esters of IAA, IPA, IAyA, ILA and ANTH spotted to give a range from 0.05 to 1.0 μg. Fraction III was also brought up in methanol (100 μl) and spotted on a Q-6 plate. A standard solution of TAM and IAMD was spotted to give a range from 0.05 to 2.0 μg. The plate was then developed in solvent A to a distance of 12 cm. After complete drying with warm air, the plate was sprayed and quantitated in the same manner as previous fractions. Fraction IV was brought up in 200 μl of methanol containing 0.5% concentrated hydrochloric acid and assayed either by one-dimensionl TLC using the same system (with appropriate standards) as employed for fraction III or by two-dimensional TLC. For two-dimensional TLC, 25–50% of the methanol solution was spotted on an activated Q-6 plate and developed for 10 cm in solvent A. After complete drying with warm air, the plate was turned 90° and run 8 cm in solvent B. Determination of TRP and KYN, after spraying with Ehrlich's reagent, was semi-quantitative, as only a single aliquot was spotted, and a limited number of standards from previous plates were available for comparison.

RESULTS

All of the fractions isolated were clean enough to allow TLC quantitation of the compounds listed (see tables) to less than 1 μg/g wet weight feces, with an accuracy of ± 10%.

TABLE II

RECOVERIES OF SUPPLEMENTED COMPOUNDS

For abbreviations of compounds, see Table I.

Compound	*Recovery**	*Fraction***
IND	95 ± 7	I
SKAT	89 ± 5	I
TOL	96 ± 7	I
IAA	92 ± 7	II
IPA	88 ± 6	II
IAyA	88 ± 10	II
ILA	98 ± 3	II
ANTH	92 ± 10	II
IAMD	48 ± 3	III
TAM	97 ± 3	III
KYN	87 ± 9	IV
TRP	87 ± 9	IV

* Values are reported as per cent mean recoveries ± standard deviation for three to five recovery tests.

** See Fig. 1.

TABLE III

TRYPTOPHAN METABOLITES IN RAT FECES*

Compound	*Range*	*Mean*	*Compounds not detected*	*Upper limit***
IND	4.5 –7.8	6.4	TOL	0.20
SKAT	0 –0.78	0.20	IAyA	0.20
IAA	0.53–3.5	2.1	ILA	0.20
IPA	0.34–4.5	2.5	ANTH	0.10
TAM	0.17–1.7	0.67	IAMD	0.10
TRP	0.92–1.8	1.5	KYN	0.30

* The values are μg/g wet weight feces collected from seven one-year-old male Sprague-Dawley rats fed Purina Rat Chow. They are based on four to six pooled collections and are corrected for 100% recovery.

** The upper limits were based on the limit of detection for a given compound and the amount of background color present.

Most of the pigments and fats contained in the ethanol homogenate went into the ether fraction. By evaporating this layer over distilled water, and subsequent filtration, most of the interfering substances were removed with little loss of the indoles. Amberlite XAD-2 has been used extensively in urine extractions[29–32]; here the resin allows desalting and concentration of fraction IV, with good recovery of TRP and KYN. Fractions II and III were free of interfering substances after simple liquid–liquid extractions.

Recoveries of the compounds listed in Table II were determined by adding either 10 or 100 μg of each compound to a 3.0-g fecal homogenate (60 ml ethanol). Recoveries were calculated by subtracting endogenous values for the same sample from the apparent recovery. In general, the compounds were obtained in high yield. The completeness of the ethanol homogenization was checked by resuspending the centrifuged residue in 95% ethanol, 80% ethanol or pH 7 buffer, and subsequent work-up in a manner similar to that for the initial homogenate. No detectable amounts of the compounds of interest were found in these additional homogenates.

Amounts of tryptophan metabolites detected in rats on a chow diet are given in Table III.

DISCUSSION

The method presented has been used to establish quantitatively the normal levels of the compounds listed (Table III) in the feces of rats on a chow diet. Previous methods for detecting indoles in rat feces[16,20] were unable to detect the low levels of indoles present in feces of rats on a normal diet.

Although an accuracy of $\pm$ 10% is claimed for quantitation by visual inspection of the thin-layer chromatogram, proper (or fortuitous) selection of standard quantities for comparison often allows a greater degree of confidence. The chromatogram can most probably be quantitated by densitometry if greater precision is desired.

An average value of 45 μg TRP/g wet weight feces has been reported[22] using an enzymatic assay of an uncentrifuged aqueous homogenate, compared to our finding of 1.5 μg/g. However, enzymatic determinations of tryptophan in centrifuged

aqueous homogenates give results in agreement with our determinations[33]. The tryptophan appears to be largely bound to the particulate matter contained in an ethanolic or aqueous homogenate.

Preliminary findings indicate large increases in the concentrations of IND, SKAT, IAA and TA (approx. 10×, 100×, 2× and 5×, respectively), and an abscence of IPA in the feces of rats on a high meat diet. Studies to determine further the effect of diet on tryptophan metabolites in feces are now in progress.

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