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Note

Chromatographic separation of tryptophan metabolites on a polyamide column

MAMDOUH Y. KAMEL and RAGAA R. HAMED
National Research Centre, Department of Biochemistry, Cairo (Egypt)
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Different chromatographic methods for the separation of tryptophan metabolites from different biological materials have recently been reviewed[1]. During the last decade, different column chromatographic techniques using sialic acid[2], Amberlite IR-120[3], Sephadex G-25[4], Sephadex G-10[5], Dowex-1[6], and DEAE cellulose[7] have been described. These methods have the disadvantage of being limited in application to either a small number of tryptophan metabolites or a specific group of indole derivatives[2–8]. However, a method for the separation of tryptophan and 15 metabolites by ion-exchange chromatography using Sephadex QAE-A 25 and carboxymethylcellulose in a continuous system has been reported[9].

In this communication a method is described for the separation, identification and quantitative determination of tryptophan metabolites by chromatography on polyamide columns. It has been applied successfully for the separation of tryptophan metabolites in *P. notatum* culture media.

MATERIALS AND METHODS

The indole derivatives were purchased from Sigma (St. Louis, Mo. U.S.A.) and polyamide resin from Wölm (Eschwege, G.F.R.). Formylanthranilic acid was prepared from anthranilic acid and 98% formic acid according to the method described by Zentmyer and Wagner[10].

Samples and chromatographic procedures

Polyamide was washed and equilibrated with 20% ethanol before packing on to the column (30 × 0.7 cm I.D.). 10-ml amounts of standard solutions containing 4 or 5 compounds dissolved in 20% ethanol were applied at the top of the column. Unless otherwise stated the concentration of each compound was 0.1 mg/ml. The adsorbed materials were eluted with ethanol in a stepwise gradient ranging from 20 to 95% ethanol prepared in distilled water. The absorbances of the eluates were recorded at 280, 300, 330 and 360 nm.

Analysis of the column effluent

Eluates from the column were concentrated under vacuum and subjected to paper chromatographic analysis using Whatman No. 1 paper sheets. Spotted chroma-

tograms were developed using two solvent systems; propan-2-ol–ammonia–water (200:10:20) (I) and 20% KCl (II). The air-dried chromatograms were examined in UV light and spots were visualized by spraying the chromatograms with Ehrlich or sulphanilic acid spray reagents[11,12]. The extinction coefficients at 330, 280 and 300 nm were used for the quantitative determination of anthranilic, formylanthranilic and indole-3-acetic acids, respectively. Tryptophan was determined according to the method of Concon[13].

Maintainance, subculturing, and growth medium of P. notatum

Cultures were grown and maintained on Czapek Dox's medium as previously described[14]. The basal medium is supplemented with 0.37% tryptophan.

RESULTS AND DISCUSSION

The behaviour of the tryptophan metabolites considered and their eluants is shown in Table I. The tested standards were eluted in the following order; tryptamine hydrochloride, tryptophan, and kynurenine sulphate (20% ethanol); indole-3-aldehyde (early fraction of 40% ethanol); anthranilic acid (late fraction of 40% ethanol), indole-3-acetic acid (60% ethanol); indole-3-acrylic acid, formylanthranilic acid (80% ethanol); and indole-2-carboxylic acid (95% ethanol). The unresolved compounds can be easily separated and identified by paper chromatography using the solvent systems I and II (Table I). The data listed in Table I show that neutral compounds and those of low polarity tend to be eluted at lower concentrations of ethanol.

TABLE I

ELUTION OF TRYPTOPHAN METABOLITES ON A POLYAMIDE COLUMN

Compounds	*Ethanol (%)*					*1% Formic acid in ethanol*	R_F		*UV*	*Spray reagents*	
	20	*40*	*60*	*80*	*90*		*I*	*II*		*Ehrlich*	*Sulphanilic*
L-Tryptophan	+						20	49	—	Purple	—
Tryptamine HCl	+						73	43	—	Red-purple	—
L-Kynurenine sulphate	+						20	64	blue	Orange	—
Indole-3-aldehyde		+					92	43	—	Pink turning purple	—
Anthranilic acid		+					37	71	dark blue	Yellow turning orange	Yellow
Indole-3-acetic acid			+				33	65	—	Pink turning grey turning brown	—
Indole-3-acrylic acid				+			30	14	—	Brown	
Formylanthranilic acid				+			50	70	—	Orange after a time	
Indole-2-carboxylic acid					+		43	34		Pink, fading	Orange
2,3-Dihydroxy-benzoic acid						+	37	57		Negative	Pink

The methods available for colorimetric determination of tryptophan metabolites in fermentation media with or without paper chromatography lack specificity[15–17]. Also, interference by phenolic compounds present in culture media with the nitrite. Ehrlich and Salkowski reagents on the chromatograms of partially purified fractions, is well known[13,18].

The present method is used for the isolation and identification of *P. notatum* tryptophan metabolites. Four day culture filtrate was extracted with three successive portions of ethyl acetate, after acidification with tartaric acid. The extracted metabolites were separated into acidic and non-acidic fraction according to the method of Larsen[19]. The elution profile for the acidic fraction is shown in Fig. 1. The elution sequence was established by paper chromatography as described above. Although the elution sequence is reproducible, a shift in the position of anthranilic acid and indoleacetic acid peak was observed. This may be due to overloading of the column by the presence of phenolic acids *e.g.* 2,3-dihydroxybenzoic acid in this fraction. However, it was eluted by 1% formic acid in ethanol (Fig. 1). Paper chromatographic analysis of the neutral fraction revealed the presence of catechol, but no other tryptophan metabolites.

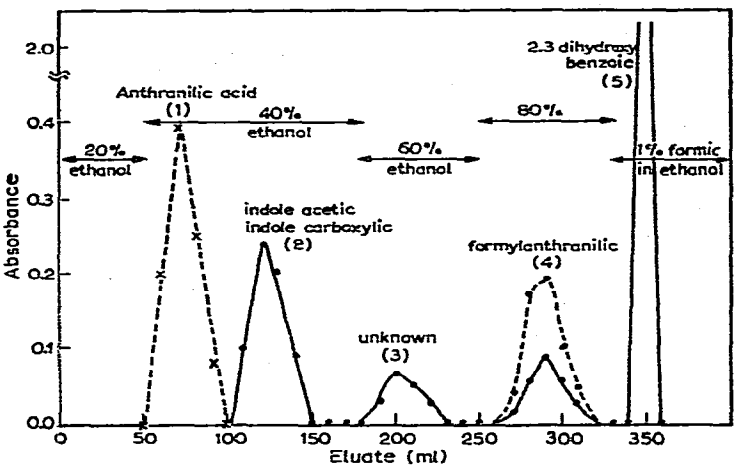
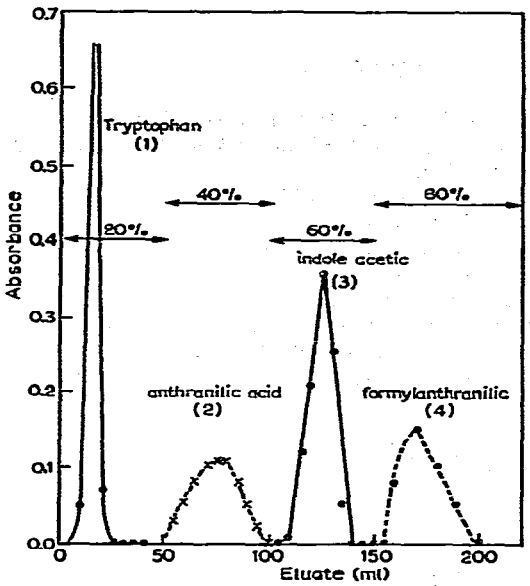


Fig. 1. Separation of tryptophan metabolites in the acidic fraction from *P. notatum* culture filtrate on a polyamide column (0.7 × 30 cm). Absorbance at: ●—● 280 nm; ●—● 300 nm; ×—× 330 nm.

For microscale quantitative determinations a mixture (50–150 μg) of tryptophan, anthranilic acid, indole-3-acetic acid and formylanthranilic acid in 7.5 ml of 20% ethanol was applied to a polyamide column (21 × 0.5 cm I.D.). A typical elution profile for four standard materials encountered in *P. notatum* culture filtrate is shown in Fig. 2. The extinction coefficients were used for quantitative determination of the microgram amounts of standard. It is obvious from the quantitative recoveries that no irreversible adsorption takes place on the column. (Fig. 3 and Table II). The method is reproducible (Fig. 3) and could be useful for the determination and qualitative identification of tryptophan metabolites in fermentation media and possibly in other biological fluids.



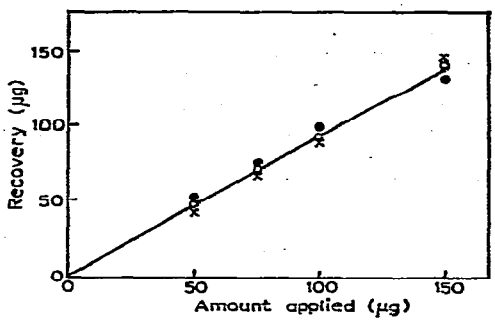


Fig. 2. Typical elution profile for standard mixture of L-tryptophan, anthranilic acid, indole-3-acetic acid and formylanthranilic acid (150 µg each) on a polyamide column (0.5 × 10 cm). Absorbance at: ●—● 280 nm; ×—× 330 nm; ⊖—⊖ 300 nm.

Fig. 3. Peak area as function of amount of sample applied to the polyamide column. ×, Tryptophan; ○, anthranilic acid; ●, formylanthranilic acid.

TABLE II

RECOVERY OF TRYPTOPHAN, ANTHRANILIC ACID AND FORMYLANTHRANILIC ACID

Concentration (µg)	*Compounds*		
	Tryptophan	*Anthranilic acid*	*Formylanthranilic acid*
50	40.6 (35.8–46.0) SE ± 2.7	47.66 (45.0–50.0) SE ± 1.06	47.23 (46.0–48.2) SE ± 0.77
75	63.4 (62.1–65.0) SE ± 0.83	71.06 (70.0–73.2) SE ± 1.06	70.4 (69.3–71.9) SE ± 0.77
100	89.06 (85.6–91.6) SE ± 1.03	91.2 (81.2–97.6) SE ± 5.08	89.3 (87.7–90.0) SE ± 3.7
150	133.3 (126.0–144.0) SE ± 3.07	136.9 (130.0–142.8) SE ± 3.73	134.5 (130.5–146.0) SE ± 2.8

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