Journal of Chromatography, 154 (1978) 122–126

CHROM. 10,881

Note

Chromatography of melanogens from urine of hamsters with transplantable melanoma

II. Thormählen-negative melanogens

Z. SCHWIPPELOVÁ, S. PAVEL, J. DUCHOŇ and B. MATOUŠ

Department of Biochemistry, Faculty of General Medicine, Charles University, U nemocnice 5, 128 53 Prague 2 (Czechoslovakia)

and

K. VULTERIN

Department of Toxicology and Forensic Chemistry, Radioisotopic Division, Na Bojišti 23, 120 00 Prague 2 (Czechoslovakia)

(First received September 30th, 1977; revised manuscript received December 17th, 1977)

Several workers have reported on the excretion of some phenolic and indolic acids in the urine of patients suffering from tumours derived from neural crest[1–8] cells (*e.g.*, neuroblastoma, melanoma, ganglioneuroblastoma and feochromocytoma). Little is known, however, about the excretion of these compounds in the urine of animals with experimental melanoma. For a qualitative study we chose chromatography on thin layers of cellulose. In addition, the distribution of carbon-14 in phenolic and indolic acids in the urine of hamsters with transplantable melanotic melanoma and those without tumours was followed.

EXPERIMENTAL

Collection of urine

Golden Syrian hamsters (*Mesocricetus auratus*) with transplantable pigmented melanoma (M-type, Bomirski, Poland) and hamsters without tumours were placed in metabolic cages and urine was collected during 24 h. The urine from individual groups of hamsters was pooled and stored at −20° for subsequent use.

Ethyl acetate extraction

A 10-ml volume of hamsters' urine was diluted with the same volume of water and acidified to pH 1 with concentrated hydrochloric acid (indicator paper). Phenolic and indolic acids were extracted twice with 15 ml of ethyl acetate. The pooled extracts were dried with anhydrous sodium sulphate and evaporated to dryness under nitrogen.

Thin-layer chromatography on cellulose plates

Thin-layer chromatography (TLC) was performed on 20 × 20 cm thin-layer

plates of cellulose [DC-Fertigplaten, PEI-Cellulose F (Merck, Darmstadt, G.F.R.) and Lucefol (Kavalier, Sklárny, Czechoslovakia)]. Ethyl acetate extracts were evaporated to dryness and the residue was dissolved in 1 ml of methanol; 20 μl of the resulting solution were applied to thin-layer plates and subjected to two-dimensional chromatography in the solvent systems (I) isopropanol–ammonia–water (8:1:1) and (II) anisole–acetic acid–water (69:30:1)[9]. The spots were made visible by reaction with diazotized *p*-nitroaniline in alkaline medium[10], Ehrlich reagent solution[11] and ammoniacal silver nitrate. For spraying with ammoniacal silver nitrate, a mixture (1:5) of 0.1 *N* silver nitrate solution and 5 *N* ammonia solution was prepared.

Application of [2-^{14}C]DOPA

For following the distribution of carbon-14 in phenolic and indolic acids in the urine of hamsters with transplantable melanotic melanoma and in healthy hamsters, [2-^{14}C]DOPA (New England Nuclear, Boston, Mass., U.S.A.) was applied i.p. to both groups of hamsters. Each hamster without tumours received $1.11 \cdot 10^5$ sec^{-1} (3 μCi) and each hamster with melanoma $2.96 \cdot 10^5$ sec^{-1} (8 μCi) of [2-^{14}C]DOPA. After the application of radioactive DOPA, the animals were placed in metabolic cages and collection of urine was begun. The urines from both groups of hamsters was separately pooled and extracted with ethyl acetate as described above.

Autoradiography

X-ray film was used for autoradiographic detection of phenolic and indolic acids. Quantitative evaluation of the radioactivity on the chromatographic plates was performed after scraping off the spots and subsequent elution with liquid scintillator (SLS-31, Spolana).

RESULTS AND DISCUSSION

TLC of ethyl acetate extracts of hamster urine revealed 13–15 phenolic and indolic acids (Figs. 1 and 2; Tables I and II). Detection with diazotized *p*-nitroaniline in alkaline medium confirmed that this procedure is suitable for the detection of the acids of interest because of the high sensitivity and the differences in colour of the individual spots. One constant difference was observed in the comparison of the ethyl acetate extracts of urine of hamsters bearing melanotic melanoma and those without tumours. In the urine from hamsters with pigmented melanoma were noted the compounds that we had identified[12] as the isomeric 5-hydroxy-6-methoxyindolyl-2-carboxylic (5H6MI2C) and 6-hydroxy-5-methoxyindolyl-2-carboxylic (6H5MI2C) acids. but they were not observed in the ethyl acetate extracts of the urine from healthy hamsters. The second difference was noted by using two types of thin-layer plates for TLC. The development of the Lucefol chromatogram took approximately half the time required with PEI-Cellulose plates, *i.e.*, the contact of phenolic and indolic acids extracted from urine with alkaline medium (solvent I) is more rapid than in the former instance. That is probably why 3,4-dihydroxyphenylacetic acid was detected only on Lucefol thin-layer plates, whereas on PEI-Cellulose plates it was destroyed (Figs. 1 and 2).

The autoradiographic study showed that most of the radioactivity was incorporated in four phenolic acids, which were identified as vanillactic acid (VLA), vanilpyruvic acid (VPA), homovanillic acid (HVA) and 3,4-dihydroxyphenylacetic acid

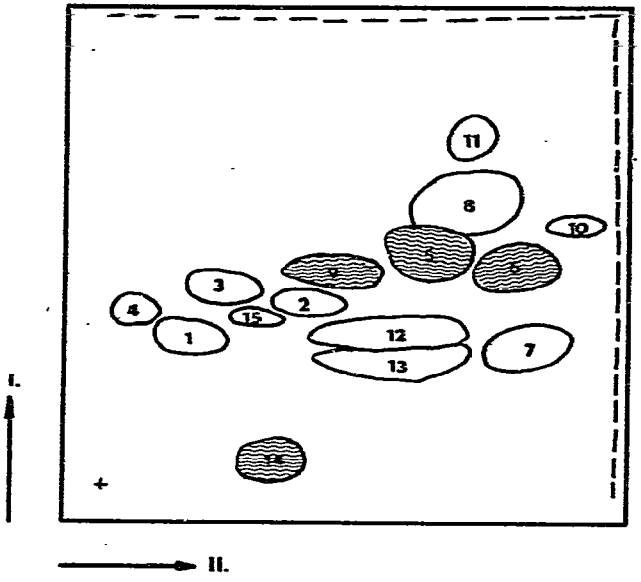

Fig. 1. Two-dimensional separation of Thormählen-negative melanogens from urine of hamsters with melanotic melanoma on thin-layer cellulose plates. The numbers of the spots correspond to the designation in Table I. The striped areas were detected by autoradiography.

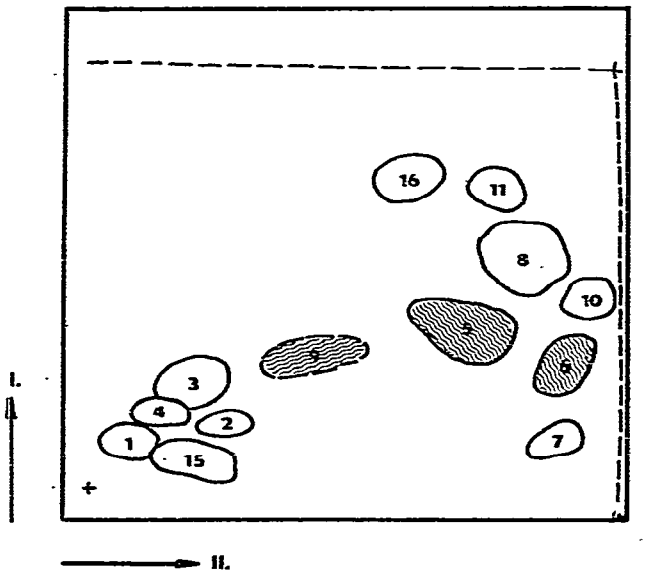

Fig. 2. Two-dimensional separation of Thormählen-negative melanogens from urine of hamsters without tumours on thin-layer PEI-Cellulose plates. The numbers of the spots correspond to the designation in Table II. The striped areas were detected by autoradiography.

(DOPAC). When a longer irradiation time was used and acids with high radioactivity were covered with a filter, a very small amount of radioactivity was detected in the case of the isomeric acids 5H6MI2C and 6H5MI2C. The amount of radioactivity

TABLE I

THIN-LAYER CHROMATOGRAPHY OF PHENOLIC ACIDS ON LUCEFOL CELLULOSE

No.	R_F		*Radioactivity*	*Colour*	*Compound**
	Solvent I	*Solvent II*			
1	0.32	0.16	—	Yellow	
2	0.40	0.42	—	Violet	VMA
3	0.42	0.23	—	Orange	*m*-HHA
4	0.37	0.06	—	Purple	*p*-HMA
5	0.50	0.61	+	Purple	VPA
6	0.46	0.81	+	Blue	HVA
7	0.29	0.80	—	Purple	
8	0.60	0.71	—	Purple	*p*-HPA
9	0.46	0.43	+	Blue	VLA
10	0.55	0.88	—	Blue-violet	
11	0.75	0.72	—	Purple	
12	0.32	0.55	—	Grey	5H6MI2C
13	0.26	0.55	—	Grey-blue	6H5MI2C
14	0.06	0.32	+	Violet	DOPAC
15	0.35	0.29	—	Purple	5-HIAA

* VMA = Vanilmandelic acid; *m*-HHA = *m*-hydroxyhippuric acid; *p*-HMA = *p*-hydroxymandelic acid; *p*-HPA = *p*-hydroxyphenylacetic acid; 5-HIAA = 5-hydroxyindolylacetic acid.

TABLE II

THIN-LAYER CHROMATOGRAPHY OF PHENOLIC ACIDS ON DC-FERTIGPLATTEN PEI-CELLULOSE F

No.	R_F		*Radioactivity*	*Colour*	*Compound*
	Solvent I	*Solvent II*			
1	0.11	0.08	—	Yellow	
2	0.16	0.25	—	Violet	VMA
3	0.26	0.19	—	Orange	*m*-HHA
4	0.18	0.13	—	Purple	*p*-HMA
5	0.44	0.65	+	Purple	VPA
6	0.31	0.82	+	Blue	HVA
7	0.12	0.81	—	Purple	
8	0.56	0.76	—	Purple	*p*-HPA
9	0.32	0.39	+		VLA
10	0.46	0.90	—	Blue-violet	
11	0.71	0.70	—	Purple	
15	0.08	0.20	—	Purple	5-HIAA
16	0.73	0.56	—	Purple	

was determined after scraping off the radioactive spots and elution with liquid scintillator. The results are presented in Table III.

The results of the incorporation of carbon-14 into the phenolic and indolic acids suggested that the isomeric acids 5H6MI2C and 6H5MI2C originate from tyrosine and DOPA, respectively.

It is very difficult, however, to explain the striking difference in the incorporation of carbon-14 into the phenolic acids on the one hand, and into the isomeric

TABLE III

DISTRIBUTION OF CARBON-14 ON THE CHROMATOGRAM OF PHENOLIC ACIDS

No.	*Compound*	*Radioactivity (cpm)*	*Relative % of radioactivity*
5	VPA	31,460.4	32
6	HVA	48,549.9	49
9	VLA	8491.4	8
12	5H6MI2C	622.4	$4.8 \cdot 10^{-3}$
13	6H5MI2C	670.4	$5.7 \cdot 10^{-3}$
14	DOPAC	10,371.4	10
Background	—	138.4	—

acids 5H6MI2C and 6H5MI2C on the other. One possibility is the later incorporation of carbon-14 into the isomeric acids, because 5H6MI2C and 6H5MI2C seem to be "later" metabolites of DOPA than VLA, VPA, HVA and DOPAC.

Nevertheless, it is necessary to consider some other possibilities. For example, the main precursor of the isomeric acids could be tyrosine and/or DOPA, which as an amino acids is contained in the peptide chain of melanoprotein. Free indole derivatives could be formed by hydrolysis using proteolytic enzymes, after the oxidation of tyrosine and/or DOPA catalysed by tyrosinase and subsequent cyclization. The next step could include methoxylation and/or conjugation with glucuronic acid or some other reactions. These possibilities, however, require further study.

REFERENCES

1 L. R. Gjessing, *J. Clin. Lab. Invest.*, 15 (1963) 463.
2 L. R. Gjessing, *J. Clin. Lab. Invest.*, 15 (1963) 649.
3 R. F. Coward, P. Smith and J. E. Middleton, *Nature (London)*, 213 (1967) 520.
4 J. Duchoň and B. Matouš, *Clin. Chim. Acta*, 18 (1967) 318.
5 J. Duchoň, B. Matouš and B. Procházková, *Clin. Chim. Acta*, 18 (1967) 318.
6 H. Hinterberger, A. Freedman and J. Bartholomew, *Clin. Chim. Acta*, 18 (1967) 377.
7 H. Hinterberger, A. Freedman and J. R. Bartholomew, *Clin. Chim. Acta*, 39 (1972) 395.
8 M. L. Voorhess, *Cancer*, 26 (1970) 146.
9 A. W. Scott, J. R. L. Smith, P. Hanson and R. Robinson, *Clin. Chim. Acta*, 63 (1975) 7.
10 J. Duchoň and V. Gregora, *Clin. Chim. Acta*, 7 (1962) 443.
11 J. B. Jepson, in I. Smith (Editor), *Chromatographic and Electrophoretic Techniques*, Heinemann, London, 1960, p. 183.
12 S. Pavel, B. Matouš, J. Duchoň and E. Bubnová, *Neoplasma*, 25, No. 3, in press.