Journal of Chromatography, 82 (1973) 127–136

CHROM. 6575

CIRCULAR THIN-LAYER CHROMATOGRAPHY FOR DIAGNOSIS AND FOLLOW-UP OF NEURAL CREST TUMOURS*

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SUMMARY

A brief review of the modern concepts of catecholamine metabolism is given. The diagnostic significance of the determination of these compounds, their precursors and catabolites in the urine of patients with neural crest neoplasias (chromaffin and neurogenic tumours of the sympathetic nervous system, malignant melanoma) is emphasized. As quantitative determinations of these metabolites by ion exchange and/or gas chromatography are delicate and time consuming, we have developed circular thin-layer chromatographic techniques. These methods are adequate but simple enough to be used in the clinical laboratory and permit the biochemical diagnosis of such tumours.

INTRODUCTION

In 1950, Engel and von Euler[1] first reported on the possibility of diagnosing pheochromocytomas by quantitative determinations of epinephrine and norepinephrine in urine. Their paper, as well as the elucidation of the biosynthesis[2–5] and catabolism[6–9] of catecholamines in the following years, formed the basis for the biochemical detection and differentiation of a number of tumours that originate from elements of the neural crest. Before examining this topic in more detail, a brief review of the normal catecholamine metabolism is necessary in order to establish the information that can be obtained by quantitative determinations of catecholamines and catecholamine catabolites in blood and urine.

In the human organism, particularly in certain parts of the brain, in postganglionic sympathetic neurons and in chromaffin cells, three catecholamines, *i.e.* dopamine, norepinephrine and epinephrine, are synthesized. These compounds originate from tyrosine (Fig. 1). Tyrosine, after absorption from the peripheral blood into cells that are capable of synthesizing catecholamines, is transformed into dopa and dopamine, a process which is catalyzed by two enzymes, the tyrosine hydroxylase and the dopa decarboxylase. In some cells, *e.g.*, those in certain parts of the central nervous system, the catecholamine synthesis is thereby terminated. In other cells,

* This work was supported by the Swiss National Foundation for Scientific Research.

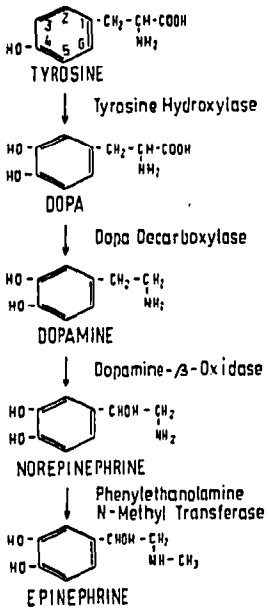

Fig. 1. Biosynthesis of catecholamines.

however, dopamine migrates into special subcellular organelles, the so-called granulated or storage vesicles, where it is hydroxylated by dopamine-β-oxidase at the β-carbon atom of the side-chain. With the norepinephrine formed by this mechanism, the catecholamine synthesis in adrenergic sympathetic neurons and part of the chromaffin cells is terminated. In most of the chromaffin cells, which are virtually all located in the adrenal medulla, a considerable fraction of the norepinephrine is finally transformed into epinephrine by the phenylethanolamine-N-methyl transferase.

In conclusion, it can be stated that in the adrenergic system, in addition to dopamine, mainly norepinephrine is synthesized, whereas in the adrenal medulla, in addition to dopamine and norepinephrine, mainly epinephrine is formed. Catecholamines are not only synthesized in the organs mentioned above but are also, according to momentary physiological requirements, continuously released into the surrounding medium and may therefore reach the systemic circulation. It follows that either the amount of epinephrine found in the blood or the urinary excretion rate of epinephrine can be taken to reflect the biological activity of the adrenal medulla, whereas the level of norepinephrine in blood or urine is related to the activity of adrenergic nerves.

As we now know, the catecholamines formed in the organism are not only released and finally excreted in the urine, but are also continuously catabolized, partly even before entering the circulation. Two enzymes (Fig. 2) are involved in this catabolism, catechol-O-methyl transferase (COMT) and monoamine oxidase (MAO). Catechol-O-methyl transferase, which is present mainly in the liver and the kidneys, methylates the hydroxyl group in the 3-position of the catechol nucleus and thereby leads to the synthesis of 3-methoxytyramine from dopamine, of normetanephrine from norepinephrine and of metanephrine from epinephrine. By determination of

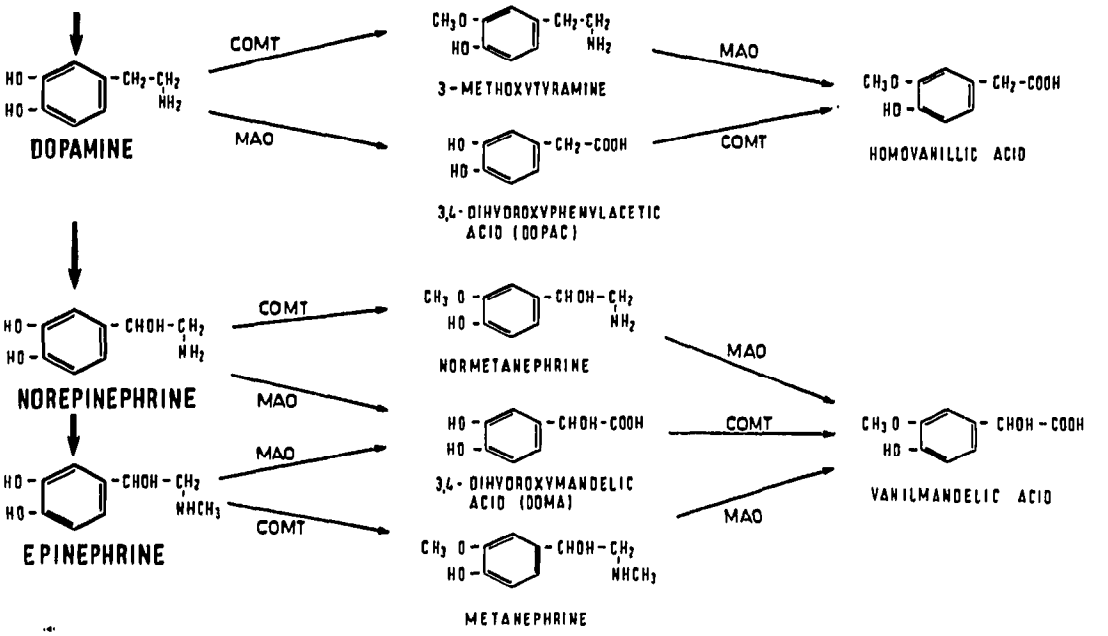

Fig. 2. Alternative metabolic pathways of catecholamines. Aldehyde intermediates, glycol derivatives and certain other metabolites are omitted. Broad arrows, major pathways; thin arrows, minor pathways. COMT = catechol-O-methyl transferase; MAO = monoamine oxidase.

these methoxy derivatives, the amounts of dopamine, norepinephrine and epinephrine that entered the blood can be estimated. The oxidative deamination effected by the monoamine oxidase, on the other hand, owing to the predominant oxidation of the intermediate aldehydes, leads mainly to the formation of catechol acids, *i.e.* 3,4-dihydroxyphenylacetic acid (dopac) from dopamine, and 3,4-dihydroxymandelic acid (doma) from norepinephrine and epinephrine. Because virtually only monoamine oxidase is present in certain organs, particularly in the sympathetic nervous system, the determination of these acids permits an estimation of the intraneuronal degradation of catecholamines. Because methoxy derivatives are also substrates for the monoamine oxidase and because catechol acids are substrates for the catechol-O-methyl transferase, homovanillic acid (HVA) is finally formed from 3-methoxytyramine and 3,4-dihydroxyphenylacetic acid, and vanilmandelic acid (VMA) from normetanephrine, metanephrine and 3,4-dihydroxymandelic acid. These phenolic acids therefore represent the final breakdown products of dopamine or norepinephrine and epinephrine, respectively. As these two catabolites emerge from various metabolic pools, they do not reflect particularly well the sympatho-adrenal activity, but give valuable information on the total amount of dopamine or norepinephrine and epinephrine synthesized in the organism.

The elucidation of the catecholamine metabolism and the development of techniques for the quantitative assessment of catecholamines, their precursors and catabolites have enabled a number of facts to be established that are not only of theoretical but also of practical interest. An important aspect of this development is the possibility of detecting and differentiating certain neuroectodermal tumours in

TABLE I

URINARY EXCRETION OF DOPA, CATECHOLAMINES AND THEIR METABOLITES IN PATIENTS WITH NEUROECTODERMAL TUMOURS

Single tick: most often and strongly increased; double tick: frequently and moderately increased; dash: normal.

Metabolite	*Pheochromocytoma*	*Neuroblastoma*	*Ganglioneuroma*	*Malignant melanotic melanoma*
Epinephrine	√	—	—	—
Norepinephrine	√	√√	(√√)	—
Metanephrine	√	—	—	—
Normetanephrine	√	√√	(√√)	—
Vanilmandelic acid	√	√	(√√)	—
Dopamine	—	√	—	—
3-Methoxytyramine	—	√√	—	—
3,4-Dihydroxyphenylacetic acid	—	√	—	—
Homovanillic acid	—	√	—	—
Dopa	—	√√	—	√
3-Methoxytyrosine	—	√√	—	√√
Vanillactic acid	—	√√	—	√√

the laboratory[10–14]. These tumours, as a rule, lead to an increased excretion of catecholamines and/or their metabolites. In addition, particular and characteristic excretion patterns permit the differentiation of these neuroectodermal neoplasias (Table I), as follows:

(1) Pheochromocytomas, which arise from chromaffin cells, produce a clearly increased excretion of epinephrine and/or norepinephrine, metanephrine and/or normetanephrine and vanilmandelic acid in the urine.

(2) In patients with neuroblastoma and ganglioneuroblastoma, tumours that arise from undifferentiated elements of the sympathetic system, the excretion of epinephrine and metanephrine is normal and that of norepinephrine is usually increased only moderately. In contrast, the urinary output of dopamine is always clearly higher than in normal children of the same age. Furthermore, the excretion of vanilmandelic acid, 3,4-dihydroxyphenylacetic acid and homovanillic acid is increased in most patients, that of normetanephrine, 3-methoxytyramine and dopa in many patients and that of the dopa catabolites, 3-methoxytyrosine and vanillactic acid, in some of these patients.

(3) On the other hand, ganglioneuromas, which consist of differentiated ganglionic cells, are generally not associated with an increased excretion of catecholamines and derivatives. In rare cases, however, particularly in those with hypertension, chronic diarrhoea or other symptoms, the urinary output of norepinephrine, normetanephrine and vanilmandelic acid is increased.

(4) Finally, as dopa is not only a precursor of the catecholamines but also of melanin, it is not surprising to find that patients with malignant melanotic, but not patients with amelanotic melanomas, frequently excrete abnormal amounts of dopa and, occasionally, of its catabolites, 3-methoxytyrosine and vanillactic acid.

Because reliable determinations of catecholamines and their metabolites necessitate laborious and delicate column chromatographic–fluorimetric or gas chro-

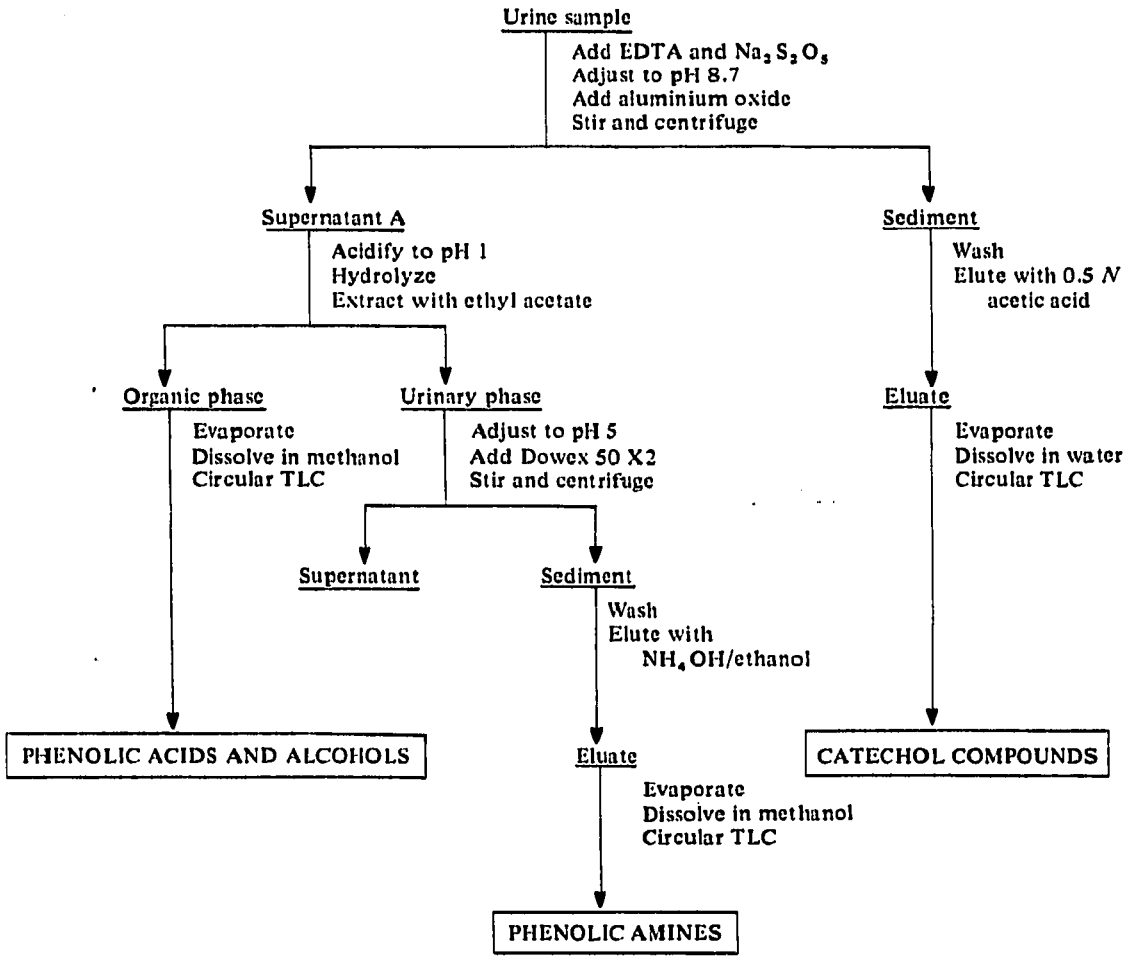

Fig. 3. Outline of procedures for the separation of catechol compounds, phenolic amines, phenolic acids and alcohols.

matographic procedures, we have developed thin-layer methods that are adequate but simple enough to be used for diagnostic purposes in the clinical laboratory. The procedures described below are outlined in Fig. 3.

CIRCULAR THIN-LAYER CHROMATOGRAPHY OF URINARY CATECHOL COMPOUNDS

To 10 ml of urine, acidified to pH 1–2 with concentrated perchloric acid during collection, 0.5 ml of 1% sodium metabisulphite and 0.5 ml of 0.2 *M* EDTA solution are added. The pH is then adjusted to 8.7 with 5 *N* K_2CO_3 solution. After addition of 0.5 g of aluminium oxide, the mixture is stirred for 10 min and centrifuged. The supernatant (supernatant A), which can be used for the determination of further metabolites, is discarded. The sediment is washed three times with 10 ml of a solution

TABLE II

CHROMATOGRAPHIC PROPERTIES OF SOME URINARY CATECHOL COMPOUNDS

Compound	R_F	*Fluorescence colour*
Dopa	0.26	Light yellow
Norepinephrine	0.33	Yellow
Epinephrine	0.40	Yellow–brown
Dopamine	0.42	Light yellow
3,4-Dihydroxyphenylacetic acid	0.81	Light yellow

containing 1% of $KHCO_3$, 0.01% of $Na_2S_2O_5$ and 0.002 *M* EDTA, adjusted to pH 8.7 with 5 *N* K_2CO_3 solution and extracted three times with 7 ml of 0.5 *N* acetic acid. The extract is evaporated at 37°. The residue is dissolved in 1 ml of distilled water and 20 μl of this solution is subjected to circular chromatography on plates coated with microcrystalline cellulose as sorbent. After development for 3–4 h at room temperature in the solvent system *n*-butanol–acetic acid–water (4:1:1), the plates are dried, sprayed with ethylenediamine–potassium ferricyanide solution[15], heated at 50° for 30 min and examined under ultraviolet (UV) light (366 nm). R_F values and fluorescence colours are given in Table II.

As a rule, only trace amounts of fluorescent substances are found in the urine of normal individuals by using this technique (Fig. 4). In patients with malignant

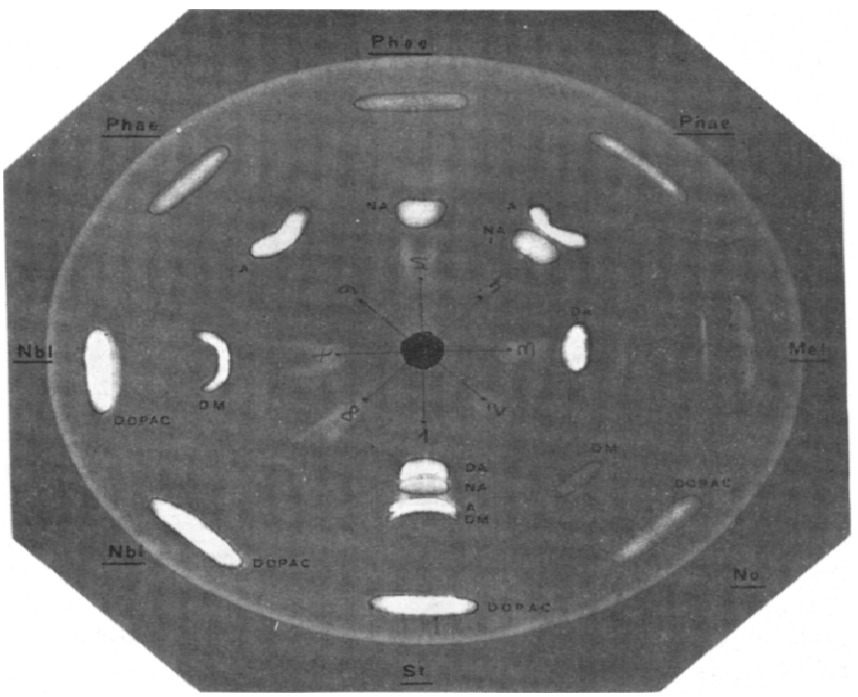

Fig. 4. Circular thin-layer chromatogram of catechol compounds. St = Standard mixture of dopa (DA), norepinephrine (NA), epinephrine (A), dopamine (DM) and 3,4-dihydroxyphenylacetic acid (DOPAC); No = normal urine; Mel = urine of patients with malignant, melanotic melanoma; Phae = urine of patients with pheochromocytoma; Nbl = urine of patients with neuroblastoma.

melanotic melanoma, the dopa is often increased. In patients with pheochromocytoma, different patterns are found: either epinephrine or norepinephrine, or both, may be increased. In patients with neuroblastoma, clearly visible bands of 3,4-dihydroxyphenylacetic acid are always seen; in most cases, in addition, bands of dopamine and in some patients bands of norepinephrine appear.

CIRCULAR THIN-LAYER CHROMATOGRAPHY OF PHENOLIC ACIDS AND ALCOHOLS

A 5-ml volume of urine or of supernatant A is adjusted to pH 1 with HCl, hydrolyzed for 30 min at 100° and extracted three times with 5 ml of ethyl acetate. The combined extracts are evaporated and dissolved in 0.5 ml of methyl alcohol. Of this solution, 5 μl are applied to the plates described above, run for approximately 4 h at room temperature in the solvent system isopropyl alcohol–aqueous NH_3–water (8:1:1) and air dried. The metabolites are made visible by spraying with cold diazotized *p*-nitroaniline solution[10]. The R_F values and the colours of the most frequently encountered compounds are given in Table III.

TABLE III

CHROMATOGRAPHIC PROPERTIES OF SOME PHENOLIC ACIDS AND ALCOHOLS IN HUMAN URINE

Compound	R_F	*Colour*
Vanillic acid	0.33	Violet
5-Hydroxyindoleacetic acid	0.37	Red
Vanilmandelic acid	0.44	Violet
Homovanillic acid	0.51	Blue
m-Hydroxyphenylacetic acid	0.62	Pink
o-Hydroxyphenylacetic acid	0.86	Pink–Violet
3-Methoxy-4-hydroxyphenylethanol	0.91	Blue

As seen in Fig. 5, in addition to trace amounts of other substances, only barely detectable bands of vanilmandelic acid and homovanillic acid are found in the urine of normal individuals. In patients with pheochromocytomas, an isolated and usually very striking vanilmandelic acid band is observed. In patients with neoroblastoma, an impressive homovanillic acid band, most often associated with a striking band of vanilmandelic acid and/or 3-methoxy-4-hydroxyphenylethanol, is seen.

CIRCULAR THIN-LAYER CHROMATOGRAPHY OF PHENOLIC AMINES

For the chromatographic separation of phenolic amines, 5 ml of urine or supernatant A, both adjusted to pH 1 and hydrolyzed for 30 min at 100°, or of the urinary phase that remains after extracting with ethyl acetate (see above), can be used. The pH is adjusted to 5 with 5 *N* K_2CO_3 solution, 0.5 g of Dowex 50 X2 (100–200 mesh, H^+ form) is added and the mixture is stirred vigorously. After centrif-

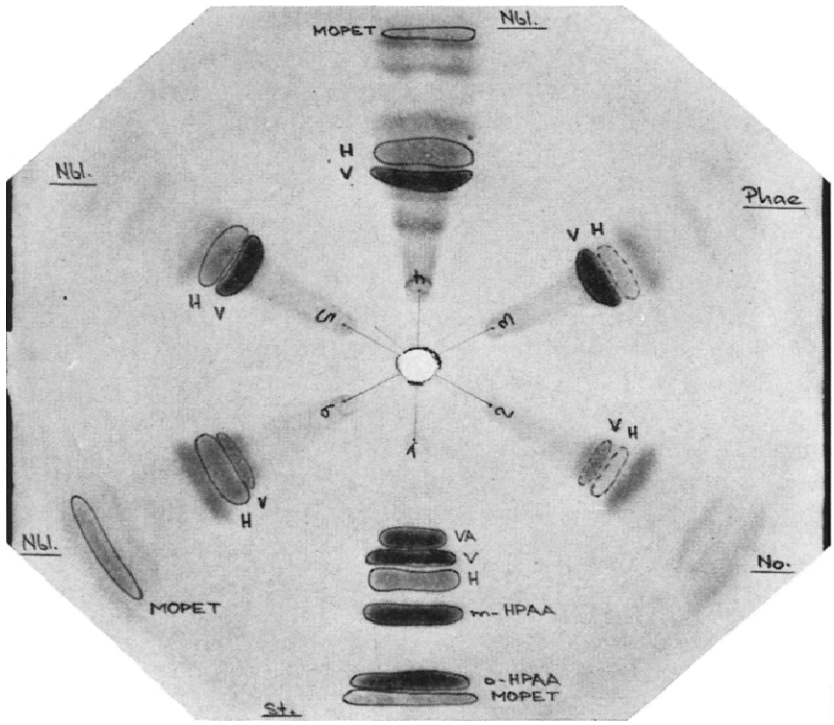

Fig. 5. Circular thin-layer chromatogram of phenolic acids and alcohols. St = Standard mixture of vanillic acid (VA), vanilmandelic acid (V), homovanillic acid (H), *m*-hydroxyphenylacetic acid (*m*-HPAA), *o*-hydroxyphenylacetic acid (*o*-HPAA) and 3-methoxy-4-hydroxyphenylethanol (MOPET); No = normal urine; Phae = urine of a patient with pheochromocytoma; Nbl = urine of patients with neuroblastoma.

ugation, the supernatant is discarded. The residue is washed three times with 0.5 ml of 0.1 *M* sodium acetate solution and is then eluted four times with a solution of 1 *N* ammonia in 65% ethanol[16]. The eluate is evaporated and dissolved in 1 ml of methanol, and 40 μl of the resulting solution are subjected to chromatography and developed as described above. R_F values and the colour reactions observed are given in Table IV.

On chromatograms of urines from normal individuals (Fig. 6) only a few, if any, barely detectable substances are observed by this method. In patients with pheochromocytomas, metanephrine and normetanephrine are found if the tumour pro-

TABLE IV

CHROMATOGRAPHIC PROPERTIES OF SOME PHENOLIC AMINES IN HUMAN URINE

Compound	R_F	*Colour*
Normetanephrine	0.61	Violet
Compound X	0.64	Violet
Octopamine	0.66	Red
3-Methoxytyramine	0.70	Blue
Metanephrine	0.78	Violet
p-Sympathol	0.86	Red

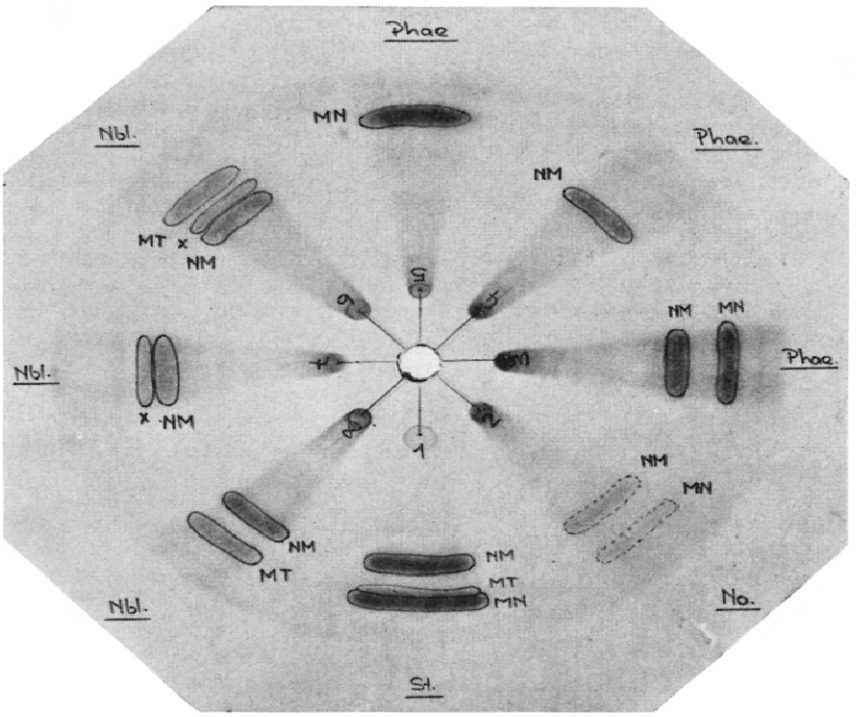

Fig. 6. Circular thin-layer chromatogram of phenolic amines. St = Standard mixture of normetanephrine (NM), 3-methoxytyramine (MT) and metanephrine (MN); No = normal urine; Phae = urine of patients with pheochromocytoma; Nbl = urine of patients with neuroblastoma.

duces both epinephrine and norepinephrine. If only one of the latter is produced either metanephrine or normetanephrine is observed. In patients with neuroblastoma, in addition to a clearly visible band of normetanephrine that is always present, increased amounts of 3-methoxytyramine and of another as yet unidentified metabolite (band X) are frequently seen.

It is evident that not only by more laborious methods, but also by those described in this paper, several tumours of neuroectodermal origin can be detected and differentiated. Furthermore, as the growth of these tumors correlates fairly well with the amount of catecholamines and catecholamine derivatives excreted, these techniques permit the evolution of such tumours to be followed adequately and enable the efficacy of therapy to be assessed objectively.

ACKNOWLEDGEMENT

I wish to thank Miss U. Pfister for her expert technical assistance.

REFERENCES

1 A. Engel and U. S. von Euler, *Lancet*, (1950) 387.
2 P. Hagen, *J. Pharmacol. Exp. Ther.*, 116 (1956) 26.
3 D. J. Demis, H. Blaschko and A. D. Welch, *J. Pharmacol. Exp. Ther.*, 117 (1956) 208.

4 S. Udenfriend and J. B. Wyngaarden, *Biochim. Biophys. Acta*, 20 (1956) 48.
5 McC. Goodall and N. Kirshner, *J. Biol. Chem.*, 226 (1957) 213.
6 J. Axelrod, *Science*, 126 (1957) 400.
7 J. Axelrod, *Physiol. Rev.*, 39 (1959) 751.
8 R. J. Wurtman, *Catecholamines*, Little, Brown & Co., Boston, 1962.
9 I. J. Kopin, *Pharmacol. Rev.*, 16 (1964) 179.
10 W. v. Studnitz, *Scand. J. Clin. Lab. Invest., Suppl.*, 12 (1960).
11 W. v. Studnitz, H. Käser and A. Sjoerdsma, *N. Engl. J. Med.*, 269 (1963) 232.
12 H. Käser, *Schweiz. Med. Wochenschr.*, 96 (1966) 258.
13 K. Türler and H. Käser, *Clin. Chim. Acta*, 32 (1971) 41.
14 H. Käser, *Helv. Paediatr. Acta, Suppl.*, 29 (1972).
15 S. Takahashi and L. R. Gjessing, *Clin. Chim. Acta*, 36 (1972) 369.
16 Y. Kakimoto and M. D. Armstrong, *J. Biol. Chem.*, 237 (1962) 208.