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Professor Candin Liteanu on his 100th anniversary

RAPID SCREENING METHOD FOR THE DIAGNOSIS OF ADRENAL TUMORS

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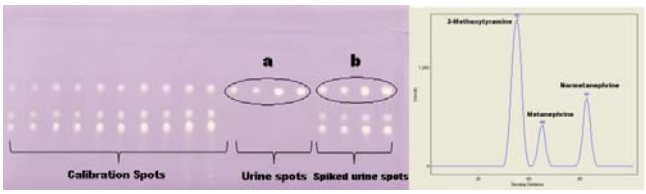
The increased excretion of normetanephrine, metanephrine and 3-methoxytyramine in urine is indicative of the presence of pheochromocytoma, neuroblastoma or paragangliomas, therefore the development of new methods has appeared to be of great importance in the early screening of these diseases. Consequently, measurement of these catecholamine metabolites in spiked urine specimens, has been carried out by applying a rapid and convenient thin-layer chromatography method with DPPH· radical detection and specialized software for quantitative TLC analysis.
The obtained results showed recovery rates between 97.96% and 103.48% for all of the investigated compounds, thus making this method suitable for routine screening of adrenal tumors associated with over-production of catecholamines and hyper-excretion of their metabolites.



INTRODUCTION

In rare cases the medulla of the adrenal glands produces a tumor, originated in the chromaffin cells, known as phoechromocytoma, occurring most often during young-adult to mid-adult life. Around 80-85% of pheochromocytomas arise from the adrenal medulla and 15-20% arises from extra-adrenal chromaffin tissues and these tumors are called paragangliomas. Neuroblastomas are primitive tumors that derive from the same blastic precursor as in pheochromocytomas, and are distributed along the sympathetic nervous system.[1]

Because the main symptom of pheochromocytoma is the secretion of high amounts of catecholamines, mostly norepinephrine, epinephrine and in some cases dopamine, the diagnosis of this tumor relies on the evidence of catecholamine over-production in plasma or urine. The catecholamines are metabolized within the chromaffin cells to metanephrines, an intra-tumoral process that occurs independently of catecholamine release by these tumors.[2]

Usually the metabolites of norepinephrine and epinephrine, normetanephrine (NMN) and metanephrine (MN), are found in relatively small amounts in healthy humans. The increased excretion of these metabolites is indicative of the disease, but does not completely rule out other diseases which may cause the same excretion values.

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Therefore, the measurements of fractionated metanephrines (normetanephrine and metanephrine measured separately) in the plasma or urine specimens provide a better diagnostic sensitivity than the measurement of plasma or urine catecholamines.[3, 4]

In most laboratories dopamine and its metabolite 3-methoxytyramine are not routinely tested in every case of suspected pheochromocytomas/paragangliomas because cases of pure dopamine secreting tumors are rare. However, these tests can be useful in some cases, especially metastatic disease, as metastatic tissue lacks the mature enzymes necessary for the synthesis of catecholamines[5] and elevated levels of 3-methoxytyramine has been suggested to be a very sensitive marker of malignant tumor when compared to the assays for plasma or urinary catecholamine metabolites.[5,6]

In the last years, a number of liquid chromatography-tandem mass spectrometry assays have been developed for a sensitive measurement of normetanephrine, metanephrine and 3-methoxytyramine in plasma or urine, in order to accurately diagnose these diseases.[4,7-15] Although these methods have given sensitive and reliable results, it is well known that the acquisition of this equipment is highly costly and also, in most cases, the preparation of biological samples involves many complicated and time consuming steps. Therefore the development of new methods has appeared to be of great importance in the early screening of pheochromocytoma and other adrenal tumors.

Because of our previous work involving catecholamines and their acidic metabolites[16-18] we have developed a screening method, by applying the same methodology (thin-layer chromatography with DPPH· radical detection) in case of normetanephrine, metanephrine and methoxytyramine.

EXPERIMENTAL

Reagents

Analytical-grade methanol and formic acid (85%) were purchased from Chemical Company (Iași, Roumania); 2,2-diphenyl-1-picrylhydrazyl free radical (95%) (DPPH free radical) was from Alfa Aesar (Karlsruhe, Germany) and the citrate buffer solution (pH = 3.00) was obtained from Sigma-Aldrich (Steinheim, Germany). The analytical purity DL-Normetaphrine hydrochloride, DL-Metanephrine hydrochloride and 3-methoxytyramine hydrochloride used in this study were obtained from Sigma-Aldrich (Steinheim, Germany).

Equipment and Software

The standard and sample volumes were applied using a semi-automatic sample applicator for qualitative and quantitative TLC analysis (Linomat 5, Camag). The evaluation of the chromatographic plates was made using BioDit Thin Layer Chromatography (TLC) Scanner for the image acquisition and the specialized TLC software ImageDecipher-TLC version 2.0 (BioDit Technology, Co.) for the digital image processing and quantification of compounds on the TLC plates. The limit of detection and the limit of quantification (LOD and LOQ) were calculated using SMAC (Statistical Methods in Analytical Chemistry) software and Statistica 8.0 (StatSoft, Inc., Tulsa, USA) software package was used for statistical data treatment.

Standard and Sample Preparation

The stock solution, containing a mixture of normetanephrine, metanephrine and 3-methoxytyramine was prepared with a concentration of 10 mg/mL, and this solution was next used after proper dilution for all analyses. For the calibration curve, seven different volumes, with a concentration between 0.10–1.30 mg/spot for each compound, of standard stock solution were spotted on the chromatographic plates in duplicate.

The applicability of the method was assessed by analyzing spiked urine samples, collected from two healthy volunteers (a male and a female). The urine was collected on a period of 24 hours and the volunteers were subjected to a controlled diet 72 hours before the collection of urine. Thus, the volunteers were advised to avoid the consumption of coffee, tea, chocolate, fruits (especially bananas) and vanilla flavored products 72 hours before sampling, also avoid smoking, physical and mental stress during sampling.

After the urine collection, the sample preparation involved a few simple steps. Therefore, a portion of 10 mL was diluted two times with methanol, filtered through a syringe filter NYLON 0.45µm (25 mm Ø, Teknokroma), spiked with known quantities of stock solution of metanephrines and directly used for the TLC analysis.

HPTLC Procedure

Simultaneous separation of normetanephrine, metanephrine and 3-methoxytyramine was performed using the chromatographic conditions optimized and applied in our previous work involving similar compounds.[16–18] Thus, the RP-18W$_{F254S}$ chromatographic plates [20cm×10cm, Merck, Darmstadt, Germany) and a mixture of citrate buffer (pH=3.00) : methanol : formic acid (96:4:5 v/v/v)] was used for a good separation of the investigated compounds. The TLC developing chamber was saturated for 15 min before development of the plates. In all cases, the plates were developed to a distance of 8 cm from the start. After the chromatographic separation, the plates were dried in the oven for 1 h at 65°C, cooled at room temperature and spayed with a 0.02% DPPH solution prepared in ethanol. The TLC conditions yielded to a good separation (Fig. 1) of the investigated compounds ($R_{F(Normetanephrine)}$ = 0.63, $R_{F(Metanephrine)}$ = 0.46 and $R_{F(3\text{-}methoxytyramine)}$ = 0.38) which appeared on the chromatographic plates as bright-white spots against a purple background.

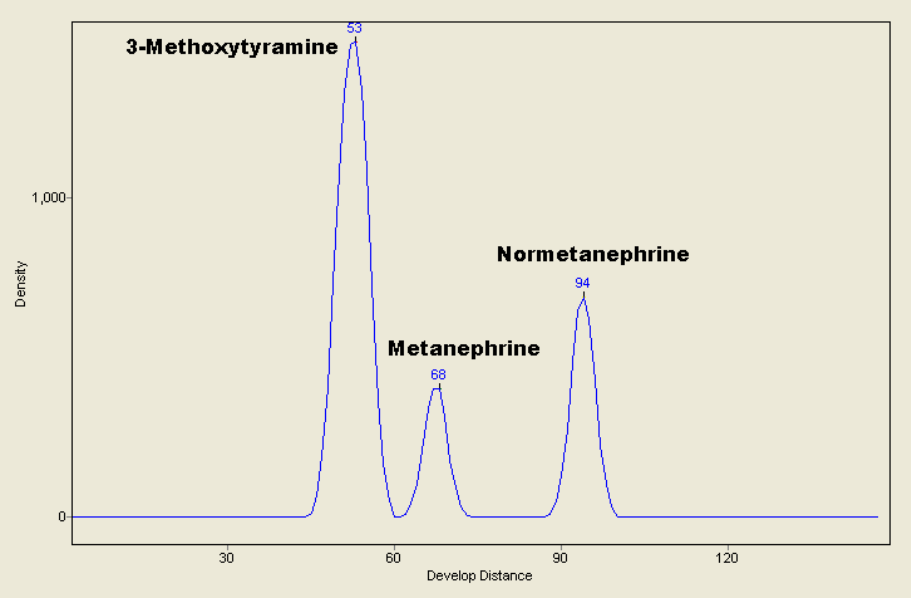

Fig. 1 – 2D chromatogram of investigated compounds.

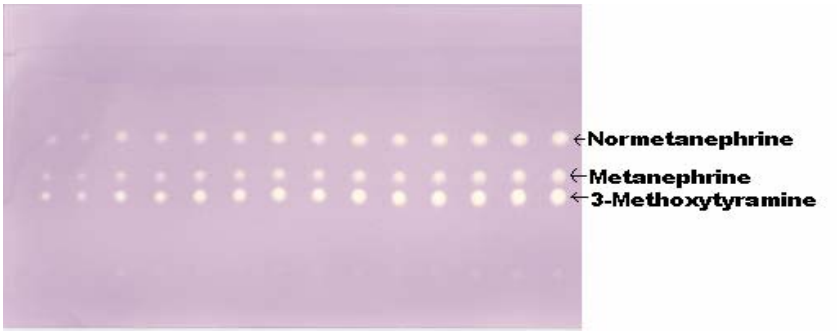

Fig. 2 – Image of the TLC plate after DPPH visualization procedure.

RESULTS AND DISCUSSION

Image Analysis and Chromatograms Processing

As described in our previous work, in order to obtain the images of the chromatographic plates (bmp file format), the methodology involves, after the separation and detection of the chromatographic spots, scanning the plates using a TLC Scanner in visible mode. Next the raw colored images of the chromatographic plates were imported directly from the scanner and for an accurate determination they were inverted and processed after their conversion into green scale using the ImageDecipher-TLC software (Fig. 2).

Method Validation

The HPTLC method was validated in terms of linearity, precision, accuracy, limits of detection and quantification, for the mixture of normetanephrine, metanephrine and 3-methoxytyramine.

For the calibration procedure seven increasing volumes of the stock solution were applied on the chromatographic plate and the calibration function was constructed for the investigated compounds, by plotting the measured peaks area versus applied amount of compound, between 0.10 and 1.30 µg/spot in all cases. The linearity was characterized by the linear range, the regression equation and the coefficient of determination value (R^2), which was greater than 0.997 for all of the investigated compounds (Table 1). The limits of detection and quantification were calculated using the SMAC software based on the confidence bands of the calibration curve, and the values obtained for these parameters were in range of 0.045 – 0.048 µg/spot for LOD and 0.087 – 0.093 µg/spot for LOQ, respectively (Table 1).

Table 1

Method evaluation parameters (linearity range, regression equation, coefficient of determination (R^2), limit of detection (LOD) and limit of quantification (LOQ))

Compound	Linearity range (μg/spot)	Regression equation	R^2	LOD (μg /spot)	LOQ (μg /spot)
Normetanephrine	0.10-1.30	y = 11077x + 504.91	0.9973	0.048	0.093
Metanephrine	0.10-1.30	y = 9857.8x + 598.49	0.9975	0.046	0.090
Methoxytyramine	0.10-1.30	y = 12911x + 3467.60	0.9977	0.045	0.087

Table 2

Accuracy and precision of the TLC – image processing method

Compound	Added amount (μg/spot)	Intra-day precision RSD (%)	Inter-day precision RSD (%)	Added amount (μg /spot)	Mean recovery (%)
Normetanephrine	0.30	4.67	4.61	0.20	106.59
	0.70	2.57	2.34	0.60	103.34
	1.10	1.98	1.81	1.00	100.87
Metanephrine	0.30	4.59	2.69	0.20	105.43
	0.70	3.72	3.57	0.60	103.02
	1.10	1.99	1.98	1.00	99.13
Methoxytyramine	0.30	2.83	1.94	0.20	106.54
	0.70	1.52	1.36	0.60	104.31
	1.10	1.13	0.93	1.00	100.91

The precision of the method, characterized as intra-day and inter-day precision, was expressed as relative standard deviation (RSD %) and determined at three concentration levels (0.30, 0.70 and 1.10 μg/spot). The intra-day precision was assessed by analyzing six replicate spots for each concentration and the inter-day precision was assessed by performing the analysis by the same analyst during a period of 5 days. The values obtained for these parameters were between 1.13% and 4.67% for intra-day and between 0.93% and 4.61% for inter-day precision, respectively (Table 2). The accuracy of the method, expressed as recovery, was investigated for standard solutions by analyzing 3 replicate spots for each of the compounds, at three concentration levels (0.20, 0.60 and 1.00 μg/spot). In this case the results (Table 2) were included in range of 105.43%–106.59%, 103.02%–104.31%, and 99.13%–100.91% at low, intermediate and higher concentrations, respectively.

Analysis of normetanephrine, metanephrine and 3-methoxytyramine in urine samples

The applicability of the method was tested on human urine samples that were spiked with known quantities of normetanephrine, metanephrine and 3-methoxytyramine standards.

For this study, urine samples were collected from two volunteers (as described in the standard and sample preparation chapter) and in order to determine the concentration of the investigate compounds, the samples were spiked with normetanephrine, metanephrine and 3-methoxytyramine, because in case of healthy subjects the concentration of these compounds in urine is very low (under the detection and quantification limit of this method).

Therefore, firstly chromatographic plates were spotted with standard mixture of the investigated compounds, in order to obtain a calibration curve, and also with urine and spiked urine. As it can be observed in Fig. 3 metanephrine and 3-methoxytyramine were confirmed by a good separation resolution on the basis of their retention by comparison with the spots of the standards samples, but at the same R_F as of normetanephrine (R_F = 0.63) it can be observed that an interfering compound is present in the urine sample.

Based on the fact that our method cannot detect normetanephrine in urine of healthy subjects, another chromatographic system was employed to demonstrate this theory. Thus, standard solutions of metanephrine, normetanephrine, urine and spiked urine samples were spotted on TLC-silica gel 60 chromatographic plates and then they were developed using a mobile phase consisting of phosphate buffer : methanol 80:20 (v/v). In Fig. 4 is shown the plate described before, after spraying it with 0.02% DPPH ethanolic solution, and it can be observed that the investigated compounds are no longer separated, but the unidentified compound is now well separated from the rest, thus demonstrating that it isn't normetanephrine.

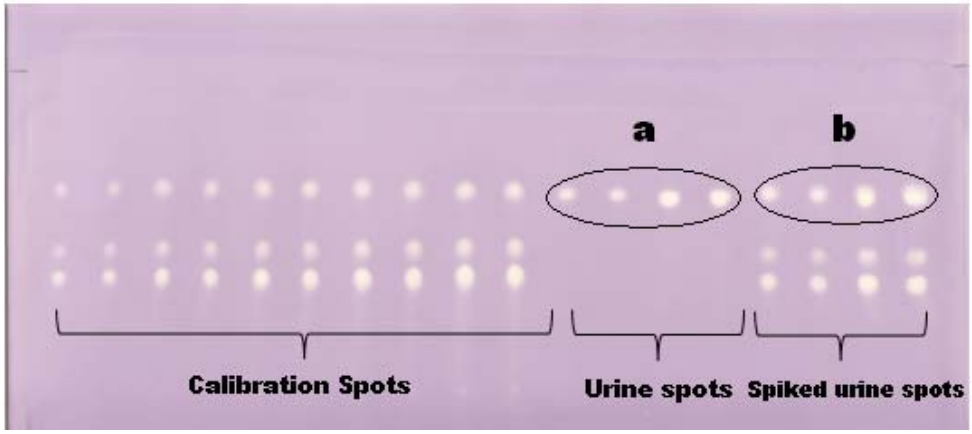

Fig. 3 – Image of the chromatographic plate presenting the analysis of urine and spiked urine samples.

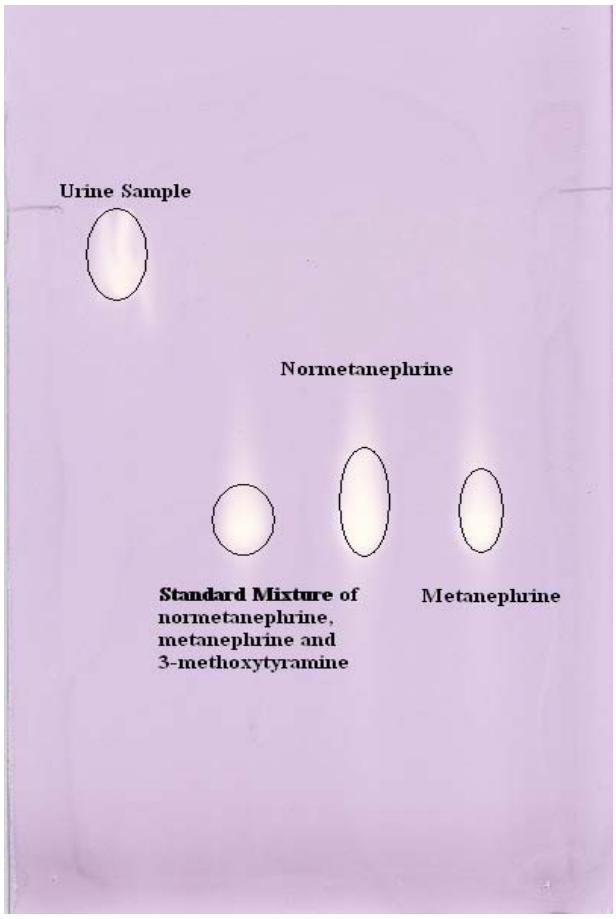

Fig. 4 – Image of the plate developed in modified chromatographic conditions.

Table 3

Recovery results of spiked urine sample

Compound	Fortified value (μg/spot)	Found value (μg/spot)	Mean recovery (%)
Normetanephrine	0.400 0.800	0.394 0.784	98.49 97.96
Metanephrine	0.400 0.800	0.405 0.797	101.24 99.59
Methoxytyramine	0.400 0.800	0.419 0.828	104.86 103.48

Based on the linear regression equation obtained for all the investigated compounds, we were able to quantify the amount of metanephrine and 3-methoxytyramine directly using the area of the respective compound separate from the spiked urine sample and in case of normetanephrine the quantification was made using the area obtained by the difference between the area of the spiked urine spot (Fig. 3 – spots a) and the area of the unidentified compound (Fig. 3 – spots b). The results (Table 3) show recovery rates between 97.96% and 103.48% for all of the investigated compounds.

CONCLUSIONS

In conclusion, the HPTLC method assisted by specialized software and DPPH detection, developed in our previous work, was successfully applied for the quantification of three catecholamine metabolites that are associated with adrenal tumors when hyper-secreted in urine.
Using this method, metanephrine and 3-methoxytyramine were determined from spiked urine samples without interferences from the matrix, and although some unidentified compound was interfering with normetanephrine, we were able to quantify it with good recoveries.

The simple sample preparation, inexpensive equipment and short analysis time are grounds that recommend this method for the rapid screening of pheochromacytomas and other adrenal tumors.

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REFERENCES

1. I. Ilias and K. Pacak, *Horm. Metab. Res.,* **2005**, *37*, 717-721.
2. J. M. Pappachan, D. Raskauskiene, R. Sriraman, M. Edavalath and F. W. Hanna, *Curr. Hypertens Rep*., **2014**, *16*, 442-455.
3. J. W. Lenders, K. Pacak and M. M. Walther, *JAMA*, **2002**, *287*, 1427-1434.
4. A. M. Sawka, R. Jaeschke, R. J. Singh and W.F. Young Jr., *J. Clin. Endocrinol. Metab.,* **2003**, *88*, 553-558.
5. A. Van Berkel, J. W. Lenders and H. J. Timmers, *Eur. J. Endocrinol.,* **2014**, *170*, 109-119.
6. G. Eisenhofer, D. S. Goldstein and M. M. Walther, *J. Clin. Endocrinol. Metab.,* **2003**, *88*, 2656-2666.
7. M. J. Whiting, *Ann. Clin. Biochem.,* **2009**, *46*, 129-136.
8. W. H. de Jong, E. G. de Vries and B. H. Wolffenbuttel, *J. Chromatogr. B*, **2010**, *878*, 1506-1512.
9. M. Peitzsch, D. Pelzel and S. Glöckner, *Clin. Chim. Acta*, **2013**, *418*, 50-58.
10. T. Hasegawa, K. Wada and E. Hiyama, *Anal. Bioanal. Chem*., **2006**, *385*, 814-820.
11. X. He, J. Gabler, C. Yuan, S. Wang, Y. Shi and M. Kozak, *J. Chromatogr. B*, **2011**, *879*, 2355-2359.
12. E. Grouzmann and F. Lamine, *Best Prac. Res. Cl. En.,* **2013**, *27*, 713-723.
13. G. Eisenhofer, J. W. M. Lenders, H. Timmers, M. Mannelli, S. K. Grebe, L. C. Hofbauer, S. R. Bornstein, O. Tiebel, K. Adams, G. Bratslavsky, W. M. Linehan and K. Pacak, *Clin. Chem.,* **2011**, *57*, 411-420.
14. B. J. Petteys, K. S. Graham, M. L. Parnás, C. Holt and E. L. Frank, *Clin. Chim. Acta*, **2012**, *413*, 1459-1465.
15. Z. D. Clark and E. L. Frank, *J. Chromatogr. B*, **2011**, *879*, 3673-3680.
16. I. A. Sima, D. Casoni and C. Sârbu, *Talanta,* **2013**, *114*, 117-123.
17. I. A. Sima, D. Casoni and C. Sârbu, *J. Liq. Chromatogr. R. T.,* **2013**, *36*, 2395-2404.
18. D. Casoni, I. A. Sima and C. Sârbu, *J. Sep. Sci.,* **2014**, *37*, 2675-2681.