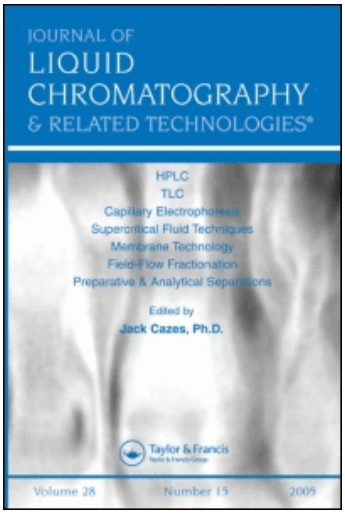

Journal of Liquid Chromatography & Related Technologies
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713597273

The Determination of Urinary Metanephrine and Normetanephrine by High Performance Liquid Chromatography with Ultraviolet Absorbance Detection

P. Kaplan[a]; M. Tejral[a]; L. Nentwich[a]
[a] Biochemistry Department, Faculty of Medicine, Masaryk University, Brno, CSFR

To cite this Article Kaplan, P. , Tejral, M. and Nentwich, L.(1992) 'The Determination of Urinary Metanephrine and Normetanephrine by High Performance Liquid Chromatography with Ultraviolet Absorbance Detection', Journal of Liquid Chromatography & Related Technologies, 15: 14, 2561 — 2579
To link to this Article: DOI: 10.1080/10826079208017202
URL: http://dx.doi.org/10.1080/10826079208017202

JOURNAL OF LIQUID CHROMATOGRAPHY, 15(14), 2561-2579 (1992)

THE DETERMINATION OF URINARY METANEPHRINE AND NORMETANEPHRINE BY HIGH PERFORMANCE LIQUID CHROMATOGRAPHY WITH ULTRAVIOLET ABSORBANCE DETECTION

P. KAPLAN, M. TEJRAL, AND I. NENTWICH
Biochemistry Department
Faculty of Medicine, Masaryk University
662 43 Brno, CSFR

ABSTRACT

The sensitive and specific direct analysis of urinary normetanephrine and metanephrine (both free and conjugated) was achieved utilizing reversed-phase high performance liquid chromatography and UV detection. The urine specimens were hydrolysed in a boiling water bath and treated with isoamyl alcohol:toluene to eliminate interference from urinary pigments or related chemical compounds. The methoxyamines were isolated by the passage through a small ion-exchange column and then pre-concentrated by a rapid set of solvent (ethyl acetate:acetone) extractions before being injected into the chromatographic column.

Detection was achieved with UV absorbance detector at 280 nm and metanephrines were quantified by standard addition method. For both metanephrine and normetanephrine the standard curve was linear over the range of 15-300 μmol/L and practical lower detection limits were about 100 nmol/L for actual urine samples, which are not too different from those using electrochemical detector. Samples can be injected at 10-min intervals.

The values for a patient with pheochromocytoma and for other patients are given.

INTRODUCTION

Catecholamines (CA) and their metabolites play an important role in the central and peripheral nervous systems. They are released as neurotransmitters at the endings of post-ganglion sympathetic neurons and, moreover, they are formed in the adrenal medulla.

A number of diseases are related to dysfunctions of catecholamines such as Parkinson's disease, hypertension, and certain neural tumours such as pheochromocytoma and neuroblastoma which may be characterized by the release of abnormally high quantities of amines and their metabolites (1,2,3,4).

An important pathway of catecholamine inactivation lies in the O-methylation of their 3-hydroxyl groups. Major catecholamines,i.e. adrenaline (A), noradrenaline (NA) and dopamine (DA), thus form metanephrine (MN), normetanephrine (NMN) and 3-methoxytyramine (3-MT), which are secreted in urine in both free and glucuronide- or sulphate-conjugates (3,5,6). These catecholamine metabolites are detectable in the urine of healthy subjects and their elevated levels have been found to be of a diagnostic importance in neural crest tumours, particularly pheochromocytoma (7,8).

Various methods have been developed for their analysis; however some lack the necessary sensitivity and others are too complicated for routine analysis. The widely used colorimetric assay of Pisano (9) has several major limitations: it does not distinguish, nor is it entirely specific for, MN and NMN, and it requires a 24 h urine collection because sensitivity is inadequate for untimed urines having "total metanephrine" concentrations less than 100 µg/L (10,11). From further existing analytical procedures

fluorimetric and radioenzymatic assays are used (12,13) and very sensitive gas chromatography-mass spectrometry (11) which, however, requires expensive equipment.

High performance liquid chromatography (HPLC) has been in recent years explored as an alternative means of separating and measuring these compounds in urine, biological liquids, and tissues. The speed and resolution now available in HPLC combined with the elimination of troublesome derivatizing steps in gas-liquid chromatography make such a system highly attractive to the analyst. The most frequently used methods of detection are fluorimetric (14,15) and electrochemical (5,6,16-19,27) ones.

In our work we wanted to test the application of UV detection method that is used very seldom (20), and its usability for clinical practice.

MATERIALS AND METHODS

Apparatus

The HPLC system consisted of a high-pressure pump HPP 5001 and analytical column (SEPARON SIX C_{18}, 150 x 3,2 mm i.d., 5 μm particles) fitted with syringe loading injector LCI 20 were obtained from Laboratory Equipments (Prague,CSFR). A precolumn packed with silica gel (10 μm) was placed between the pump and the sample injector to protect the analytical column. The absorbance variable-wavelength detector Model LC 55 B (Perkin-Elmer,Wien,Austria) was operated at 280 nm providing 50 mV output signal per absorbance unit. The elution profiles were recorded on the chart recorder TZ-4221 and (or) integrated using CI-100 electronic integrator (Laboratory Equipments).

Reagents

For standards, NMN-HCl (Calbiochem, San Diego, CA, USA), MN-HCl, 3-MT-HCl, tyramine-HCl (T) (Sigma, St.Louis, MO, USA) and 3-methoxy-4-hydroxybenzylamine--HCl (MHBA)(Janssen Chemica, Beerse, Belgium) were used. Amberlite CG 50 II (200-400 mesh) was obtained from Serva,(Heidelberg, FRG). All the other chemicals of reagent grade were purchased from Lachema, Brno,CSFR; all the aqueous solutions were prepared from doubly distilled water.

Stock standard solutions of the amines (100 mg/L in 0,01 mol/L HCl) were stored at -30 °C. Amberlite CG 50 II was used in sodium form adjusted to pH 6,5 by acetic acid and stored at 4 °C with the addition of NaN_3. Glass columns (10 mm i.d.) were filled with this cation-exchange resin to the height of 30 mm (resin volume 2,4 ml).

Mobile phase was prepared from 0,1 mol/L NaH_2PO_4 with 1 mmol/L Na_2EDTA and adjusted to pH 4,5. After filtrating, 100 volumes of the solution and 5 volumes of methanol were mixed. The eluent was always degassed prior to use under reduced pressure.

Sample Collection and Handling

Twenty-four-hour urine specimens were obtained from healthy subject, from patients (aged 14 to 67 years) suspected for the presence of increased levels of catecholamines , most of them with essential hypertension,and from one patient with pheochromocytoma (tumour confirmed by surgery). The samples were acidified to approximately 1-2 pH and stored at 2 °C if assayed within 10 days, or at -30 °C if kept longer before analysis.

Extraction

Samples were analysed by a modification of the Shoup and Kissinger procedure (16). Urine sample (20 ml) was adjusted to pH 1, divided into four 5 ml portions and transferred to a 15-ml capped centrifuge tubes. The third and the fourth portions were supplemented with 5 µg MN and 5 µg NMN, respectively. Samples were then placed in boiling water bath for 30 min. Hydrolysed urine was cooled and 5 ml of toluene/isoamyl alcohol (3:2 v/v) solution to each portion of sample were added. After shaking for 2-3 min, samples were centrifuged briefly and the upper organic layers containing urinary pigments were aspirated and discarded. The remaining aqueous layers were diluted with 20 ml H_3BO_3 solution (0,65 mol/L), their pH were adjusted to 6,5 with 5M NaOH, and passed through a column filled with Amberlite CG 50. After washing with 10 ml distilled water, metanephrines were eluted with 5 ml of NH_3 (4 mol/L) into the glass centrifuge tube containing 2,5 g NaCl.The collected eluates were extracted twice with 4 ml of ethyl acetate/acetone (2:1 v/v) solution using a reciprocal shaker for 5 min. After centrifugation, the organic layers were separated and aliquots of 4 ml were evaporated at 40 ^{o}C under stream of nitrogen. The residues were then dissolved in 50 µl of mobile phase, vortex-mixed for 20 s and let to stand for about 20 min. The aliquots (5-10 µl) of the extracts were injected onto the chromatographic column.

HPLC-Separation and Detection

The separation of amines was performed by the isocratic elution mode at laboratory temperature and a

flow-rate of 0,5 ml/min maintained. A volume of the injected sample was usually 10 µl which was reduced to volume of 5 µl if too large amounts of metanephrines were present. Separated compounds in samples were detected at 280 nm and a detector response was registered on a chart recorder. Peaks on chromatograms of urine were identified by comparing their retention times with those of standard solutions.

The concentrations of metanephrines in a sample were calculated from the peak heights by using standard addition method. Four aliquots of each urine sample were used for the determination of both NMN and MN.

The recovery of the standard was calculated for each urine sample by the substraction of amount calculated in urine without, from the amount calculated in urine with the standard added.

The detection limit was defined as the lowest concentration of amine giving a peak three times higher than the baseline noise.

RESULTS

Fig. 1 shows a chromatogram of four synthetic standards which could be resolved within 13 min. The retention time of the initial peak (Vo) was 1,83 min, the retention times of NMN, MN, tyramine and 3-MT were 3,75, 5,75, 7,15 and 11,90 min, respectively. Peak for MHBA standard (t_R 7,30 min,is not shown) was not well resolved from tyramine peak and thus could not be used as an internal standard.

The relationships between methoxyamine concentrations and peak areas or peak heights are shown

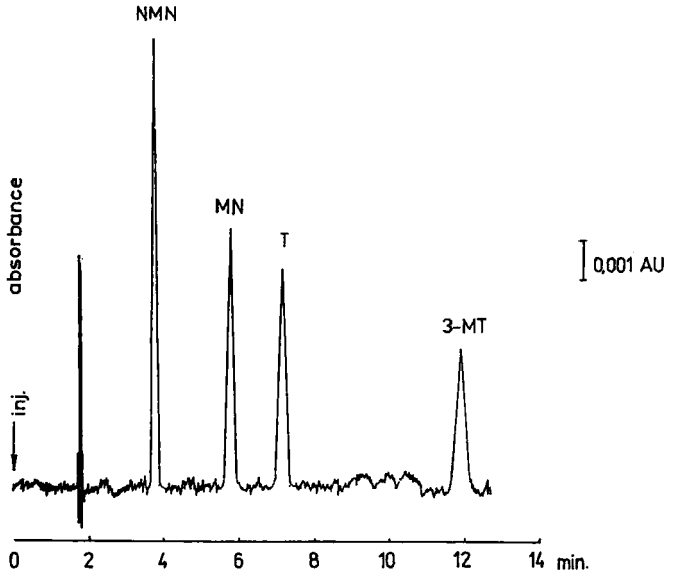

Fig. 1. Chromatogram of a standard mixture of 100 ng of NMN, MN, tyramine and 3-MT. Volume injected 10 µl, absorbance 0,02 a.u. full scale. For chromatographic condition see text.

in Fig. 2. Each point on the calibration curve represents the mean of five determinations. The responses for both compounds were found to be linear over the range investigated (0,15-2,5 nmol per 10 µl). The detector response for these substances in peak height (a.u.) vs. amount injected (nmol) was described by the following equations:

NMN: $y = 0,0206x - 0,0002$

MN: $y = 0,0138x - 0,0005$

The equations obtained for the calibration curves of peak areas (counts) vs. amount injected methoxyamines

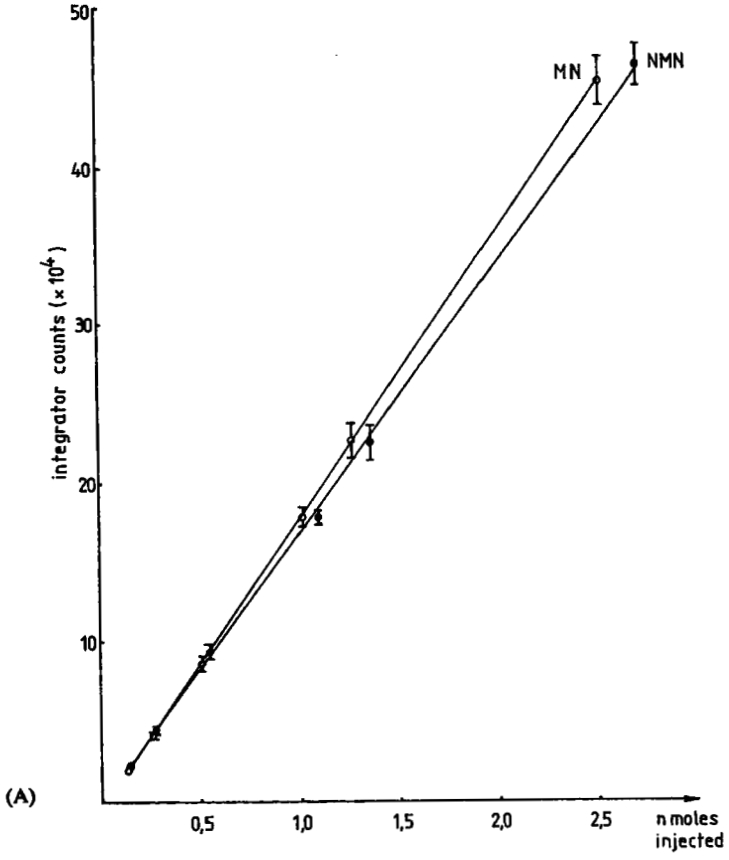

Fig. 2. Calibration curves of normetanephrine and metanephrine showing the plot of the area (A) and peak height (B) versus the injected amount of both NMN and MN.

were as follows:

NMN: y = 168 722x - 919
MN: y = 180 067x - 3672

The detection limit of NMN and MN in standard solution at a signal-to-noise ratio of 3 was approx. 60 and 100

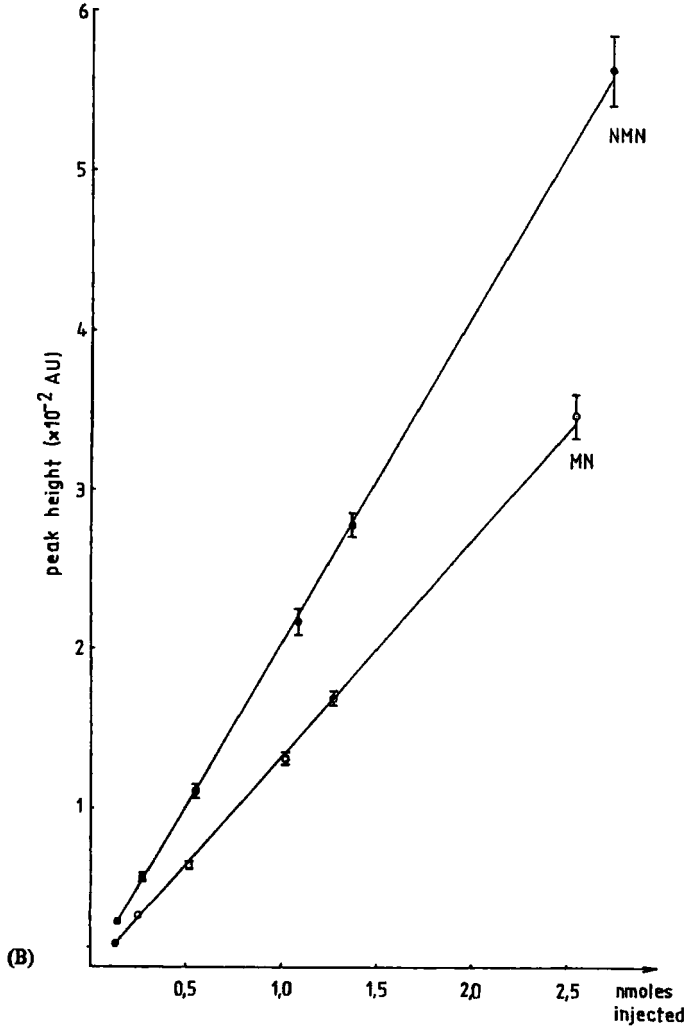

Fig. 2 (continued)

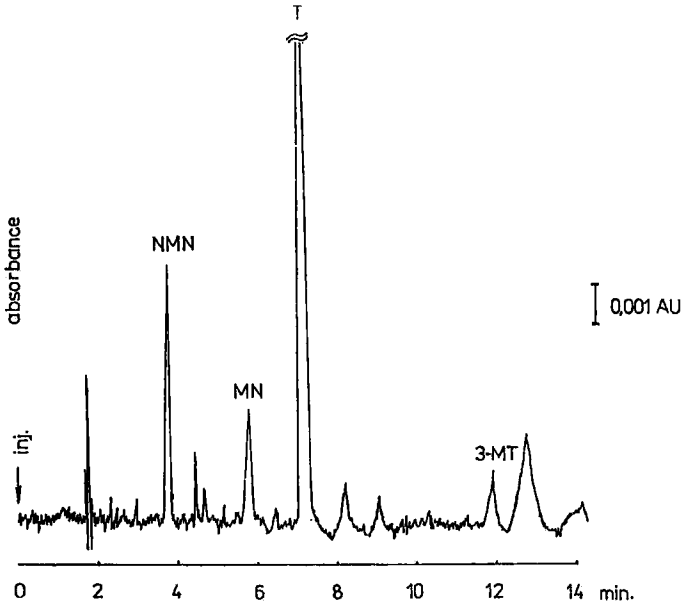

Fig. 3. Typical chromatogram obtained by injecting 10 µl of urine extract from a healthy individual. Operating conditions are as described in text.

pmol of injected metanephrines. This is equivalent to 80 and 140 pmol NMN and MN per ml urine when 5 ml of urine were extracted and 10 µl of extract injected (recoveries of both metanephrines see below).

Fig.3 illustrates a typical chromatogram that for a urine sample from a healthy 24-year-old man. In addition to NMN and MN, both tyramine and 3-MT have been identified in the chromatogram and some additional peaks which, however, did not interfere with the investigated ones.

Table 1 shows the values for five 24-h urine specimens from the same healthy subject collected over

TABLE 1

Urinary Excretion of NMN and MN by Normal Adult Individual.

Sample No	NMN*	MN*
1	1,63	1,13
2	1,37	1,40
3	1,37	1,32
4	1,39	1,91
5	2,02	1,44
Mean	1,56	1,44
SD	0,28	0,29

*Values are given in μmol/24 h.

a period of four months. Within one sample analysis in four parallel controls, the standard addition method was used and a mean metanephrine excretion in μmol/24 hours was assesed. No interferences from other basic compounds in the ammonia eluate were found in the specimens from that normal subject.

The recoveries of NMN and MN were calculated as described above. They were (means ± standard deviation) 73 ± 10% and 75 ± 12%, respectively.

The analysis of six potential pathological urine specimens are presented in Table 2. None of these patients had a confirmed diagnosis of pheochromocytoma. The data presented in Table 2 indicate that the mean values for NMN and MN in these individuals are not

TABLE 2

Urinary Excretion of NMN and MN by Six Potential Pathological Patients.

patient age	NMN*	MN*
1 33	2,73 ± 0,65	3,07 ± 0,68
2 14	4,69 ± 1,04	1,10 + 0,27
3 18	1,07 ± 0,30	0,81 ± 0,26
4 67	1,80 ± 0,54	0,31 ± 0,09
5 47	1,25 ± 0,19	0,97 ± 0,08
6 15	1,63 ± 0,24	2,53 ± 0,39

*Values are given in µmol/24 h, mean ± SD.

greater than those in normal person, except patients No.1 and No.2.

In some patients the HPLC pattern showed extra unknown peaks in all parts of the chropmatogram but no interference was observed with investigated amines. In one case, however, extraneous peaks were not well resolved from NMN peak and prevented thus its determination. (This patient was not included into the investigation).

The excretion patterns of the only patient with pheochromocytoma observed over a long term (a few years) are presented in Table 3. All urine samples had enormously elevated levels of NMN; Fig. 4 shows a

TABLE 3

Urinary excretion of NMN and MN by patient with pheochromocytoma.

Sample No	NMN*	MN*
1 preop.	37,16 ± 5,25	3,91 ± 1,51
2 postop.	12,70 ± 0,98	0,76 ± 0,18
3	63,40 ± 9,37	0,81 ± 0,25
4	84,15 ± 13,10	0,81 ± 0,10
5	86,90 ± 12,02	1,40 ± 0,50

* Values are given in µmol/24 h, mean ± SD

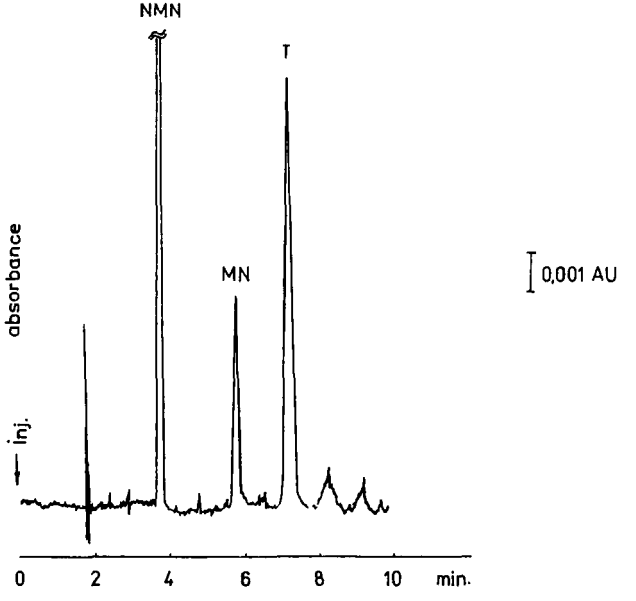

Fig. 4. Chromatogram of an urine extracted from pheochromocytoma patient. Volume injected 5 µl. Operating conditions are as described in text.

chromatogram of urine sample No. 1 collected prior to therapy (before the tumour removal).

DISCUSSION

The described method allows sufficiently sensitive determination of metanephrines in urine.

The amines were essentially analysed by the method reported by Shoup and Kissinger (16), except the bigger volume of urine and phosphate buffer at pH 4,5 in mobile phase were used.

In contrast to predominant majority of assays we use method of UV detection of amines. The absorbance detector , which is easier to handle, was operated at 280 nm. This method of detection has some advantages in comparison with that electrochemical (e.g. faster establishment of baseline, its insensitivity to changes in flow rate, temperature and mobile phase composition).

In our work we use an extraction by organic solvents and ion exchange column before HPLC. Many other authors refuse this process of sample purification because of causing sizable metanephrines losses, and they prefer the separation of these amines in the only column (3,19,21,22). As significant advantages of it they have shown the method is faster and it routinely gives higher recovery. On the other hand, however, its disadvantage is that a more complex chromatogram is obtained, which can prevent the accurate determination of the analysed substances (21). Other autors find as insufficient the using of the only column for the sample purification but extraction by organic solvents is substituted by using

the second ion-exchange column with subsequent repeated elution (17,23).

As hydrolysed urine, however, contains very often a large amount of urinary pigments we assume that additional prepurification step is necessery. Prepurificate procedure before ion-exchange chromatography removes pigments and acidic compounds and thus it prevents the decrease retention efficiency of the cation exchange column. In the beginning of our work when prepurification step was not made the recoveries of that amines (especially MN) were substantially lower.

Insufficient elution by NH_3 from ion exchange column is considered to be additional losses of metanephrines. We used 5 ml of 4 M NH_3 for elution from 2,5 ml weak cation exchange resin. Many other authors used similar volume of ammonia in the same concentration range. E.g. Brown et al.(24) shows 2,5 ml 1 M NH_3 for 1,3 ml of Bio Rex 70 resin, Bertani-Dziedzic et al. (22) used 6 ml 4 M NH_3 for amine elution from 4,5 ml of Dowex 50. On the other hand, Jouve et al.(23) eluted 2,3 ml of Bio Rex 70 by 30 ml (!) 4 M NH_3 whereas Moleman (3) recommended 4,5 ml 1 M ammonia as the optimal amount for the volume of 1,3 ml of Bio Rex.

To find out whether elution of amines from cation exchange column in our work is sufficient, we have made chromatographic analysis of the second ammonia eluate of the same urine sample. The repeated elution showed unmeasurable amounts of both amines.

In some analysed urines the HPLC pattern showed extra unknown peaks and in one case the NMN was not determined for its interference with those peaks in chromatogram. These unidentified peaks can result from pharmacotherapy in patients, which ingested, for

instance, aspirin, paracetamol, and propranolol (18). Especially some beta-adrenoceptor-blocking drugs or their metabolites with retention times near that NMN, have been reported to make quantification of this amine impossible (19,21). Also the consumption of some fruits (e.g. bananas and oranges) has been reported to result in a considerable increase in the peaks of NMN and 3-MT and additional peaks eluting just after NMN, MN and 3-MT, respectively (25).

For the quantification of analysed amines we used peak height measurement method , which is considered by some workers a less precise one (3) but, in general, that is advised in favour of peak area measurement in routine HPLC analysis (26).

The using of integrator for measuring peak area was reduced only for both metanephrine standards calibration curve constructing with about the same analytical errors.

During the development of procedure we have used standard addition method. Other authors mostly use internal or external standard method. The former one was not used because of certain interference of MHBA with tyramine. Futhermore, an internal standard can be marked by different extraction behavior and does not guarantee an improvement in the assay precision. In contrast to the latter the standard addition method is advantageous, when the recovery of metanephrines varies between the samples, but it is reproducible with the same sample (3,20).

We have assayed urine samples from eight subjects by the method described. The excreted amounts of metanephrines in seven of them was either physiological or somewhat greater if compared with values reported by other laboratories (2,16,27,28)

Pheochromocytoma is rather rare so that only one such sample has been obtained. In this case the excretion of NMN was more than fifteenfold greater compared to the normal values. After the removal of tumour there was a striking decrease in the excretion rates of NMN and MN (Table 3,sample No.2). In spite of great decrease the patient continued to excrete large quantity of NMN while MN excretion was normal. In futher time post-operation excretion rates of NMN were increased again (Table 3) due to probably multiple metastatic state. The differences between the values in this patient and other subjects are thus readily apparent.

Although this technique is used at present for the determination of urinary NMN and MN it could be used to the quantification of other biological amines such as 3-MT, tyramine and serotonine.

REFERENCES

1. Bravo E.L., and Gifford R.W., N. Engl. J. Med.,311, 1298-1303 (1984).

2. Crawford G.A., Gallery E.D.M., and Gyory A.Z., Clin. Chim. Acta, 157, 121-126 (1986).

3. Moleman P. and Borstrok J.J.M., Biogenic Amines, 3, 33-71 (1985).

4. Said R., Robinet D., Barbier C., Sartre J., and Huguet C., J.Chromatogr.-Biomed.Appl.,530, 11-18 (1990).

5. Buu N.T., Angers M., Chevalier D., and Kuchel O., J. Lab. Clin. Med., 104, 425-432 (1984).

6. Westerink B.H.C., and Kate N., J. Clin. Chem. Clin. Biochem., 24, 513-519 (1986).

7. Manager W.M., and Gifford R.W., Pheochromocytoma, Springer - Verlag, Inc., New York, 1977.

8. Bravo E.L., Tarazi R.C., Gifford R.W., Steward B.H., N. Engl. J. Med., 310, 682-686 (1979).

9. Pisano J.J., Clin. Chim. Acta, 5, 404-414 (1960).

10. Dutrieu J., and Delmotte Y.A., Fresenius` Z. Anal. Chem., 316, 505-508 (1983).

11. Canfell C., Binder S.R., and Khayam-Bashi H., Clin. Chem., 28, 25-28 (1982).

12. Kahane Z., and Vestergaard P., J. Lab. Clin. Med.,70, 333-342 (1967).

13. Dalmaz Y.,and Peyrin L., J.Chromatogr.,116, 379-394 (1976)

14. Jackman G.P., Clin. Chem., 27, 1202-1204 (1981).

15. Jackman G.P., Clin. Chim. Acta, 120, 137-142 (1982).

16. Shoup R.E., and Kissinger P.T., Clin. Chem., 23, 1268-1274 (1977).

17. Parker C.N.,Levtzow C.B., Wright P.W., Woodard L.L., and Chapman J.F., Clin. Chem., 32, 1473-1476 (1986).

18. Gupta R.N., Clin. Chem., 36, 538-540 (1990).

19. Pagliari R., Cottet-Emard J.M., and Peyrin L., J. Chromatogr.-Biomed.Appl., 563, 23-36 (1991).

20. Filser J.G., Koch S., Fischer M., and Müller W.E., J.Chromatogr., 493, 275-286 (1989).

21. Wilson S.P., Kamin D.L., and Feldman J.M., Clin. Chem., 31, 1093-1094 (1985).

22. Bertani-Dziedzic L.M., Krstulovic A.M., Dziedzic S.M., Gitlow S.E., and Cerquera S., Clin. Chim. Acta, 110, 1-8 (1981).

23. Jouve J., Mariotte N., Sureau C., and Muh J.P., J. Chromatogr., 274, 53-62 (1983).

24. Brown R.T., Kirk K.L., and Oliver J., J. Liq. Chromatogr., 9, 831-843 (1986).

25. Abeling N.G., Clin. Chim. Acta,137, 211-226 (1984)

26. Snyder L.R., and Kirkland J.J., Introduction to Modern Liquid Chromatography, John Wiley & Sons, Inc., New York, 1979.

27. Moleman P., Clin. Chim. Acta, 189, 19-24 (1990).

28. Munion G.L., Seaton J.F., and Harrison T.S., J. Surg. Res., 35, 507-515 (1986).