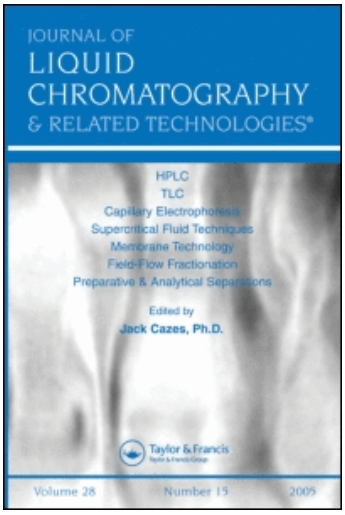

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DETERMINATION OF URINARY METANEPHRINES IN MAN USING LIQUID CHROMATOGRAPHY WITH ELECTRO-CHEMICAL DETECTION

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ABSTRACT

A procedure is developed for determining the O-methylated catecholamine metabolites, normetanephrine and metanephrine, in human urine by using liquid chromatography with electrochemical detection. Normetanephrine and metanephrine are isolated from hydrolyzed urine samples by ion-exchange chromatography on small disposable columns. They are then directly separated by reversed-phase ion-pair liquid chromatography and quantified with electrochemical detection versus a calibration curve. The chromatographic separation of these metabolites is achieved within 15 min. This method is simple, specific, sensitive and available for clinical screening of pheochromocytoma in hypertensive patients. Ranges of normal and pathological values are proposed.

INTRODUCTION

Catecholamines and their O-methylated metabolites, particularly normetanephrine (NMN) and metanephrine (MN), are normally present in human urine. Their augmentation is systematically checked to confirm, in hypertensive patients, the diagnosis of pheochromocytoma.

Identification and quantitation of these amines in biological samples have been carried out by various analytical methods. Among them, the fluorimetric assays, after periodate oxidation, are still widely used (1,2). These methods involve multiple purification steps by ion-exchange resin or organic extractions, which are time consuming and responsible, at least partially, for inaccuracy. Moreover the fluorimetric detection may be subject to interferences from dietary and drug substances.

More recently, the progress realized in the separation of biogenic amines and their related metabolites by reversed-phase ion-pair liquid chromatography with isocratic elution (3) and the use of electrochemical detection (ECD) (4) led to develop new LC-ECD methods which are more specific and sensitive. Most of these methods are concern with catecholamine determination in urine and other biological fluids, but relatively few deal with NMN and MN. Moreover, they usually include complex sample preparation steps, either by multiple solid phase purification (5) or with combined organic extraction and resin purification (6-10).

We propose an LC-ECD assay for determination of urinary NMN and MN with a simple and rapid sample preparation, which avoids extraction

or derivatization and provides specificity and sensitivity for these two substances in human urine. The described isocratic elution enables the separation of the majority of catecholamines and indoleamines and their main metabolites. This method allows to readily perform 10 to 20 determinations per day, without the use of an automatic injector.

MATERIALS AND METHODS

Chemicals

Normetanephrine hydrochloride (NMN) and metanephrine hydrochloride (MN) were purchased from SIGMA (St Louis, Mo, USA), ammoniac and hydrochloric acid from UCB (Leuven, BELGIUM) ammonium acetate, sodium hydroxyde, boric acid, monopotassium phosphate, dipotassium phosphate from MERCK (Darmstadt, GFR) ethylenediaminotetraacetic acid disodium salt (Na_2EDTA) and citric acid from FLUCKA (Buchs, SWITZERLAND). All were reagent grade.

Heptane sulfonic acid in acetic solution (PIC B7) was purchased from WATERS Assoc. (Millipore, Milford, Mass., USA) and the acidic cation exchange resin, type DOWEX AG50 Wx4 (100-200mesh hydrogen form) from BIORAD (Richmond, Ca, USA).

Equipment

The liquid chromatographic system consisted of a model 6000 A pump (WATERS Assoc.), a U-6K injector (WATERS Assoc.) and a stainless steel column (15 x 0,45 cm) packed with a reversed-phase

C18 spherical 5µm ULTRASPHERE (BECKMAN, Berkeley, Ca, USA).
No guard-column was used.
The electrochemical detector was an ELDEC 103 (CHROMATOFIELD, Marseilles, FRANCE), with a glassy carbon working electrode. Its potential was maintained at +0,8 V versus a silver/silver chloride reference electrode. The detector sensitivity was set at 5 or 1 nA full scale deflection.

Reagents, Standards and Mobile Phase

* O-methylated standard stock solutions (0,5 mg/mL free base) were prepared in 0.1 M HCl and stored at 4°C for one month. Working solutions were prepared daily by diluting these stock solutions with distilled water.

* Ammonium acetate buffer 0.4 M, pH 6.

* Borate buffer pH 8.5 : 0.06 M H_3BO_3, 0.15 M citric acid monohydrate, 0.08M $K_2HPO_4,3H_2O$. pH 8.5 with 2 M NH_4OH.

* 0.2 M EDTANa_2 pH6 with 2 M NH_4OH.

* Ion-exchange resin preparation : the cation exchange resin was used as provided by the manufacturer. No recycling step was necessary. Every week, a suspension was prepared with 25g of DOWEX in 100 ml of ammonium acetate buffer and kept at 4°C.

* The mobile phase was a binary mixture of methanol (9 volumes) with 0.1M potassium dihydrogen phosphate, 0.1 mM Na_2EDTA buffer solution (91 volumes). 1 vial of PIC B7 was added per liter of mixture. The mobile phase was accurately adjusted to pH 3.85 with

3M potassium hydroxide and filtered under vacuum through a 0,45 µm type HA filter (Millipore) before use. The flow rate was 1 mL/min.

Sample Preparation

* Urine was collected over a period of 24 hours into vessels containing 15mL of 6M HCl, and stored at 4°C until the assay.

* Hydrolysis : 5 mL of urine, standard solutions or urine control added with 0,5mL of 5 M HCl, 0.5mL $EDTANa_2$ pH6 and 4mL of distilled water were heated for 30 minutes at 100°C and cooled to room temperature. Final volume was adjusted to 20 mL with distilled water. 5mL aliquots of each sample were mixed with 0,5mL of $EDTANa_2$ and adjusted to pH 6 with 2 M NH_4OH (about 0.3mL).

* Ion-exchange chromatography : the disposable plastic columns placed on the vacuum system (BAKER) were filled up with 2.5mL of DOWEX suspension. Each pH-adjusted sample was quantitatively poured into the column. Five washings were carried out with : water (10mL), borate buffer (10mL), water (10mL, twice) and 0.1 M HCl (10mL), under vacuum aspiration at 2 mL/min. The elution was performed by 5mL of 4.5 M HCl and the eluate pH adjusted by adding 0,5mL CH_3COONH_4 (6M) and 2mL of 10 M NaOH. These samples were then injected onto the chromatographic column.

Quantitative Analysis

Calibration curves were obtained by assaying aqueous solutions of both NMN and MN (concentrations : 200, 400, 600 and 800 µg/mL)

which were carried through the entire procedure. One urinary sample, used as control, was included in each analytical batch. Quantitation was performed by measuring peak heights, and urine concentrations were obtained by intrapolation from the standard curve. No recovery correction was needed since standard solutions used for calibration curves were treated in the same conditions as the urine samples. The results were expressed as µg/L for both NMN and MN, and taking into account the diuresis, as µg/24h.

RESULTS AND DISCUSSION

Optimization of the Chromatographic Conditions

The objective of this method was to achieve the separation and detection of the O-methylated catecholamines in human urine, with minimal interference from pigments and other related compounds (norepinephrine, epinephrine, dopamine and their metabolites). Figures 1 and 2 show, respectively, chromatograms of standard solution and urine sample, both run through the entire assay procedure. Using heptane sulfonic acid and varying methanol concentration, ionic strength and pH of the mobile phase, we established the optimal conditions for a relevant separation of a standard mixture containing 15 catechol and indolaminergic compounds. The separation was achieved within 30 min. and allowed resolution of NMN and MN in less than 12 min. The pH of the mobile phase must be accurately maintained at 3.85 and, depending on the age of the column, the

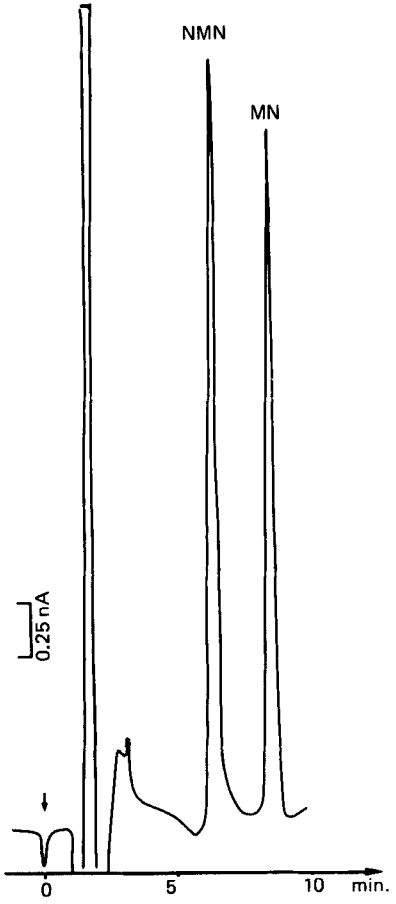

Figure 1 : Chromatogram of a Standard Solution after Dowex Resin Separation (NMN= 600µg/L, MN= 600µg/L).

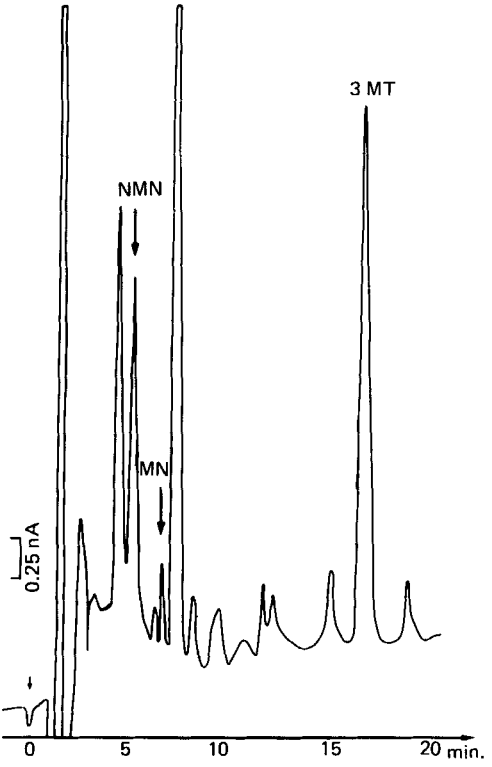

Figure 2 : Typical Chromatogram of One Urine Sample from Hypertensive Patient Without Pheochromocytoma (NMN= 300µg/L, MN= 90 µg/L). 3-MT = 3-methoxytyramine was not Quantified.

percentage of methanol has to be modified to obtain a clean resolution. The peak eluting at 17 minutes was identify as 3-methoxytyramine (3-MT).

Sample Preparation

The sulfuric acid conjugates of catecholamines and their metabolites may be hydrolysed either by heating at a strongly acid

reaction or by sulfatase preparations (11). Heating with acid was preferred since the sulfatase treatment may lead to interferent peaks.

Hydrolysis of metanephrine conjugates was complete at 100°C within 30 min. (2, 12) and there was no loss, as verified by assaying standard aqueous solutions before and after the hydrolysis step. By heating same urine samples at various temperatures, we observed that hydrolysis was not completed when water bath temperature was below 100°C (data not shown).

Cation-exchange resin type DOWEX 50 Wx4 has been used since 1960 and is satisfactory for isolation of NMN and MN from biological fluids because of its high capacity for the amines (2). However, the pH of the sample must be adjusted to 6.0 to obtain entire fixation. Both NMN and MN are completely retained by the resin and each washing step removes numerous interfering substances, without eluting the O-methylated metabolites, as demonstrated by analyzing each chromatographic fraction. The elution was performed with 5 mL of 4.5 M HCl. This volume is sufficient to obtain the greatest recovery and additional volumes would increase the final eluate volume without significant increase of recovery. The eluate pH, strongly acidic, must be adjusted before chromatographic injection.

Liquid chromatography brought further validation of this preparation procedure widely used for many years and the use of vacuum aspiration allowed to considerably reduce time-sampling to 30 min. for 10 samples.

Method Validation

Specificity of this method is achieved by use of the DOWEX resin, the liquid chromatographic separation and the electrochemical detection at + 0.8 V.

Precision : the practical detection limit of the method is about 10 µg/L for each metabolite, with 100µl injected and a signal/noise ratio > 2 at 1 nA full scale deflection.

Linearity : calibration curves obtained with aqueous standard solutions, taken through the entire hydrolysis and resin-exchange procedure were linear for both NMN and MN up to the concentration of at least 1mg/L :

NMN : intercept = 0.43 mm ; slope = 0.247 mm.L.μg^{-1} ; r=0.9995

MN : intercept = 0.76 mm ; slope = 0.260 mm.L.μg^{-1} ; r=0.9955.

Reproducibility : within-assay precision evaluated by analyzing one urine specimen ten consecutive times, showed a coefficient of variation (C.V) of 7.9 and 9.0 % for NMN and MN respectively (table I).

The between-assay precision is evaluated by analyzing several consecutive days one normal and one pathological sample and C.Vs obtained were less than 10% (table II).

Accuracy was established from recovery, supplementation and dilution tests.

The overall absolute analytical recovery was 50% for NMN and 60% for MN, both in standard solutions and urine specimens. This recovery, which is in the same range than those obtained by other methods (5, 6, 7, 12) does not alter the performance of the assay, since we

TABLE I

Within-run Precision Values Obtained after 10 Determinations of the Same Urine Sample.

Run N°	NMN (µg/L)	MN (µg/L)
1	302	92
2	367	96
3	348	94
4	298	88
5	331	96
6	310	117
7	294	85
8	340	100
9	348	100
10	306	94
mean	324,4	96,2
C.V. (%)	7,9	9,0

TABLE II

Between-run precision : values obtained for the same sample analyzed on 6 consecutive days.

Day N°	Normal urine		pathological urine	
	NMN (µg/L)	MN (µg/L)	NMN (µg/L)	MN (µg/L)
1	262	113	409	1568
2	320	95	375	1395
3	296	88	359	1301
4	320	90	387	1404
5	293	96	431	1528
6	325	96	352	1345
mean	302,7	96,3	385,5	1423,5
C.V. (%)	8,0	9,2	7,8	7,3

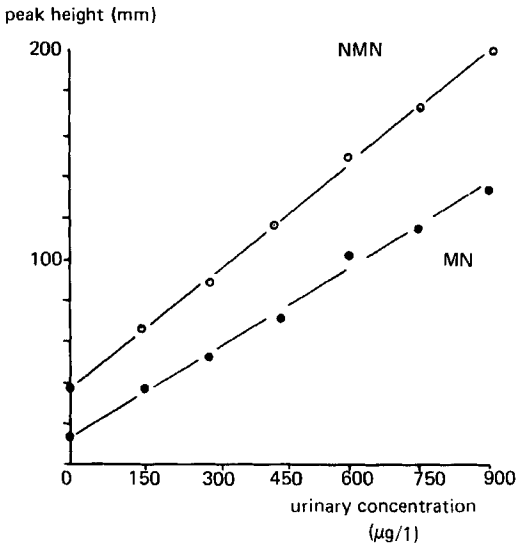

Figure 3 : Supplementation Test : Known Amounts Spiked in one Urine Sample Show Two Linear Relationships :

NMN :	direct :	int.= 0.09mm,	slope= 0.431mm.L/µg,	r= 0,9987
	calcul. :	int.= 220µg/L,	**slope= 0,994**,	r= 0,9995
MN :	direct :	int.= 12.6mm,	slope= 0.1528mm.l/µg,	r= 0,9961
	calcul. :	int.= 100µg/L,	**slope= 1,003**,	r= 0,9964

observed a good linearity and reproducibility of the standard curves and urine control between days.

By spiking urine samples with known amounts of NMN and MN, we observed no systematic relative error (figure 3).

No systematic absolute error was detected after successive dilution of one urine sample (figure 4).

Calculation of the Results

In the calculation, we did not take into account the decrease of the electrochemical response, due to the electrode pollution during the

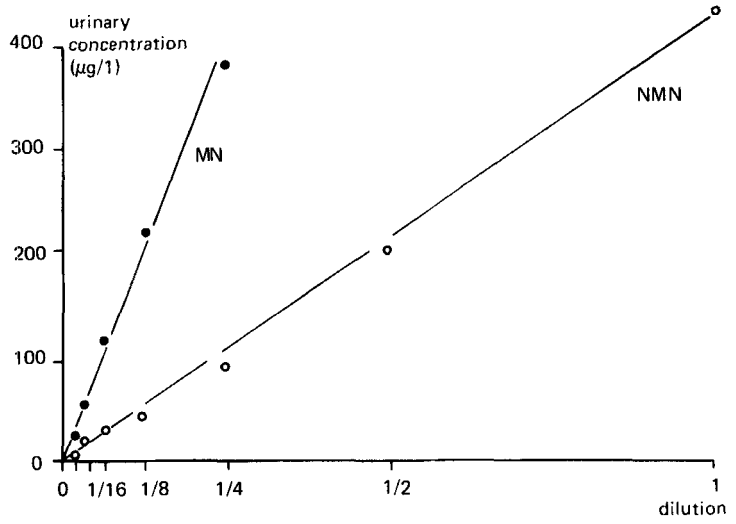

Figure 4 : Dilution Test from One Urine Sample, Shows Two Linear Relationships :

NMN :	direct :	int.= 0.09mm,	slope= 431mm.L/µg	r= 0,9987
	calcul. :	**int.= -0.39µg/L**,	slope= 1.0008,	r= 0,9995
MN :	direct :	int.= 12.6mm,	slope=1528mm.L/µg,	r= 0,9961
	calcul. :	**int.= 12.3µg/L**,	slope= 1,0005,	r= 0,9964

analysis day, as we observed that the response obtained was not significantly different, for the same injection performed at the beginning and at the end of the assay set. We did not use internal standard, for the same reasons evoked by HAEFELFINGER (13). However, the results were daily controled by assaying standard solutions and urine control sample within the batch.

Application of the Method

This method has routinely been used in our laboratory since one year, for pheochromocytoma diagnosis in hypertensive patients (1500

patients were examined and 10 pheochromocytoma detected) and for follow-up of patients post-operatively.

In order to establish mean values, 300 samples of 24h-urine collected from hypertensive patients (sex ratio =1) have been assayed. This group was chosen in preference to healthy individuals since this assay was intended to differentiate hypertensives from those with pheochromocytoma. Mean values obtained were 225 ± 114 µg/24h for NMN (range : 20-738) and 148 ± 65 µg/24h (range : 16-389) for MN. The total metanephrines excreted within 24 h was 372 ± 146 µg (range : 105-780). These results are in good general agreement with those of other assays, despite differences in subjects (healthy or hypertensive), collection period and analytical method (6, 10, 12, 14, 15). Figure 5 shows a pathological sample tracing where, in comparison with normal urine, NMN is not increased to a great extent, but MN peak is very enhanced. For determination of such samples, dilutions are needed. In patients suffering pheochromocytoma, total metanephrines excretion rate varies in a wide scale, up to 100.000 µg/24h, and the lowest pheochromocytoma we detected at this time reached 975 µg/24h.

With this method, major limitations of previous colorimetric assays have been abolished, since liquid chromatography combined with electrochemical detection assumes a convenient specificity and sensitivity and allows not only a discrimination between NMN and MN, but also a better evaluation of borderline patients. Moreover, compared with other chromatographic methods, our procedure does not require time-consuming clean-up step (6, 9, 10, 12, 15) nor

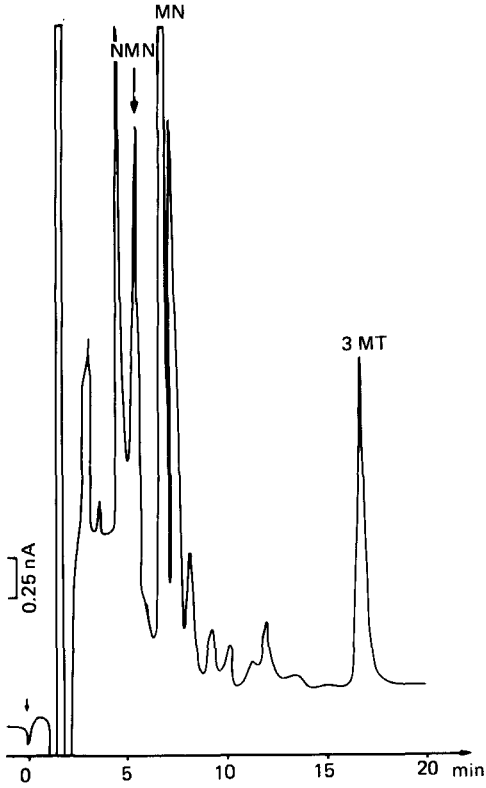

Figure 5 : Chromatogram of a Pathological Urine Sample
NMN= 360 µg/L, MN= 1300 µg/L,
Total Metanephrines = 3150µg/24h.
3-MT = 3 methoxytyramine was not quantified.

complex apparatus (e.g. mass spectrometry, 14). In emergency case, a rapid evaluation (within 2 hours) is available. With this procedure, one can routinely analyze 20 samples per day including the calibration curve.

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