Journal of Chromatography, 563 (1991) 23–36
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam

CHROMBIO. 5590

Determination of free and conjugated normetanephrine and metanephrine in human plasma by high-performance liquid chromatography with electrochemical detection

R. PAGLIARI*, J. M. COTTET-EMARD and L. PEYRIN

Université Claude Bernard, Laboratoire de Physiologie, Faculté de Médecine, 8 Avenue Rockefeller, 69373 Lyon Cedex 08 (France)

(First received April 26th, 1990; revised manuscript received August 21st, 1990)

ABSTRACT

A simple method for the simultaneous assay of normetanephrine and metanephrine (both free and conjugated) in human plasma by high-performance liquid chromatography with electrochemical detection has been developed. The hydrolysed plasma is purified on a Dowex 50 H^+ column, and the methoxylated amines are eluted with ammonia–methanol. The methoxyamines are assayed using a dual-series electrode arrangement with differential current measurement. This system greatly improves the assay specificity toward endogenous or exogenous metabolites and drugs. The high sensitivity of the method enables the determination in normal subjects of levels of normetanephrine and metanephrine.

INTRODUCTION

It has long been recognized that urinary measurements of normetanephrine (NMN) and metanephrine (MN), the direct 3-O-methylated metabolites of norepinephrine (NE) and epinephrine (E), respectively, contribute important information on adrenosympathetic activity in humans [1,2]. However, measurement of these substances in plasma may be more useful in estimating sympathetic tone not only to eliminate the confusing potential effect of renal handling on methoxyamine excretion, but also for more convenient use in ambulatory patients.

In fact, the development of assays for metanephrines in plasma has been limited by technical problems, including poor sensitivity and specificity. A radioenzymic method for plasma NMN assay involving the conversion of this amine into labelled MN by phenylethanolamine N-methyltransferase (PNMT) with tritium-labelled S-adenosylmethionine as methyl donor, was developed [3,4]. Despite its high sensitivity, this technique has the disadvantages inherent to radioenzymic assays (cost, complexity, previous preparation of purified enzyme) and it does not include the estimation of MN in plasma. On the other hand, the radioimmunoassay described by Iinuma *et al.* [5] is too complicated for routine analysis (generation of specific antibodies, use of ^{125}I-labelled synephrine) and is limited to free MN.

0378-4347/91/$03.50

This paper describes a high-performance liquid chromatographic (HPLC) method for the determination of free and conjugated NMN and MN in plasma. HPLC with electrochemical detection (ED) combines high sensitivity, specificity and rapidity for routine use.

EXPERIMENTAL

Apparatus

The chromatographic system consisted of a Kontron Model 414 pump (Zürich, Switzerland), a Rheodyne Model 7125 injection valve (Cotati, CA, U.S.A.) provided with a 100-μl loop, and a stainless-steel column (100 mm × 4.6 mm I.D.) prepared with Hypersil ODS C_{18} (particle size 5 μm). A guard column (20 mm × 2 mm I.D.) packed with pellicular ODS, 37–53 μm particle size (Whatman, Clifton, NJ, U.S.A.), was used. The ED system used was a double glassy carbon working electrode and a Chromatofield ELDEC 201 electrochemical detector (Châteauneuf les Martigues, France).

Chromatographic conditions

The analytical mobile phase consisted of 50 m*M* monosodium phosphate, 50 m*M* trisodium citrate, 2 m*M* sodium octanesulphonate, 2 m*M* disodium EDTA and 100 ml/l methanol. The final pH value was adjusted to 3.5 with 5 *M* HCl. The mobile phase was filtered before use under vacuum through a 0.45-μm cellulose nitrate filter (Sartorius, Göttingen, F.R.G.). The working potentials *versus* an Ag/AgCl reference electrode were +550 mV (V1) and +780 mV (V2), respectively, for the two electrodes. The output of the first working electrode (V1) was substracted from the output of the second working electrode (V2).

Materials

Analytical-reagent-grade chemicals were used without further purification. All solutions were prepared from deionized and glass-bidistilled water (GBW). Unless indicated otherwise, the compounds listed in in Table I were purchased from Sigma (St. Louis, MO, U.S.A.).

Sodium octanesulphonate, Dowex 50 W X2, H^+ (100–200 mesh), Amberlite CG 50, H^+ (200–400 mesh), were obtained from Fluka (Basel, Switzerland). AGMP 50, H^+ (100–200 mesh), was from Bio-Rad (Richmond, CA, U.S.A.). Sephadex G-10 (Pharmacia, Uppsala, Sweden), was prepared before use according to Westerink and Mulder [6].

All other chemicals were from Carlo Erba (Milan, Italy).

Stock solutions

Stock solutions of NMN, MN and 3-methoxy-4-hydroxybenzylamine (MHBA) (500 μ*M*) were prepared by dissolution of each compound in 0.01 *M* HCl. These solutions were prepared every month and stored at −20°C. Working solutions were obtained daily by diluting stock solutions with GBW.

TABLE I

EVALUATION OF INTERFERENCE BY SELECTED DRUGS AND METABOLITES

Mobile phase adjusted to pH 5.5 or 3.5 and the potential set at +780 mV (single working electrode) or +550 and +780 mV (double working electrode). The retention time for NMN was 6.75 min. The peak height for 2 pmol of NMN was 115 mm. N.D. = not detected.

Compound	pH 5.5		pH 3.5		
	Retention time relative to NMN	Peak height relative to NMN (+780 mV)	Retention time relative to NMN	Peak height relative to NMN	
				at +780 mV	at +550 mV (V1) and +780 mV (V2)
Amines					
NMN	1	100	1	100	100
MN	1.55	40	1.46	42	42
MHBA	2.49	21	2.29	23	23
3-MT	4.04	22	3.57	22	22
4-MT	5.92	13	5.23	13	13
Norepinephrine	0.51	346	0.41	252	N.D.
Epinephrine	0.65	313	0.56	173	N.D.
Octopamine	0.81	86	0.73	1.20	1.20
DHBA	0.96	150	0.83	134	N.D.
Synephrine	1.10	54	1.05	0.53	0.53
Dopamine	1.48	115	1.34	126	N.D.
Epinine	1.82	47	1.82	47	2
Isoprenaline	2.39	53	2.39	53	N.D.
Tyramine	2.59	21	2.59	16	16
Serotonin	4.31	49	4.31	49	–
Amino acids					
L-DOPA	0.31	951	0.26	688	N.D.
α-MethylDOPA	0.35	390	0.56	77	N.D.
3-O-MethylDOPA	0.39	339	0.60	224	–
L-Tyrosine	0.43	105	0.41	45	45
α-Methylparatyrosine	–	–	5.08	21	–
Alcohols and aldehydes					
DHPG	0.37	597	0.37	597	257
Vanillin	0.39	194	0.50	194	190
Isovanillin	0.39	297	0.50	297	290
o-Vanillin	0.39	389	0.50	387	380
MHPG	0.45	272	0.45	272	150
MHPE	1.96	67	1.96	67	67
Acids					
VMA	<0.20	N.D.	0.37	1170	1100
IsoVMA	<0.20	N.D.	0.43	614	590
Vanillic	0.33	339	1.53	159	42

(*Continued on p. 26*)

TABLE I (*continued*)

Alcohols and aldehydes	pH 5.5		pH 3.5		
	Retention time relative to NMN	Peak height relative to NMN (+780 mV)	Retention time relative to NMN	Peak height relative to NMN	
				at +780 mV	at +550 mV (V1) and +780 mV (V2)
p-Hydroxyphenylacetic	0.33	159	N.D.	N.D.	N.D.
m-Hydroxyphenylacetic	0.35	55	N.D.	N.D.	N.D.
5-HIAA	0.38	316	0.95	186	8
Homovanillic	0.39	424	1.45	177	80
Hydrocaffeic	0.47	306	1.47	165	5
o-Hydroxyphenylacetic	0.51	188	1.05	99	4
Caffeic	0.51	431	1.73	193	5
DOPAC	0.58	426	1.59	54	N.D.
Ferulic	0.86	102	0.43	52	–
Isoferulic	1.25	113	N.D.	N.D.	N.D.
Drugs					
Acebutolol	N.D.	<0.005	N.D.	<0.005	<0.005
Atenolol	N.D.	<0.005	N.D.	<0.005	<0.005
Labetalol	N.D.	<0.005	N.D.	<0.005	<0.005
Metoprolol	N.D.	<0.005	N.D.	<0.005	<0.005
Propranolol	N.D.	<0.005	N.D.	<0.005	<0.005
Sotalol	N.D.	<0.005	N.D.	<0.005	<0.005
Captopril	N.D.	<0.005	N.D.	<0.005	<0.005
Clonidine	N.D.	<0.005	N.D.	<0.005	<0.005
Acetylsalicylic acid	–	–	0.48	0.013	<0.005
Paracetamol	–	–	0.47	457	156

Preparation of the resins

Dowex 50 W X2, H^+, was purified according to Peyrin [7]. Amberlite CG 50 was prepared as described by Peyrin [7], except for the final step in which 2 *M* ammonia was used instead of borate buffer. The resin was then washed with GBW until the pH reached 7, and was ready for use. AGMP 50, H^+, was purified according to Flood and McComb [8].

Blood collection

Blood samples were obtained from fourteen normal volunteers (six men and eight women, mean age ± S.D. = 37.3 ± 9.4 years) who gave informed consent. The collection was performed in the morning at 9 a.m. The subjects had a light breakfast at 7 a.m. and were then kept recumbent. An indwelling PTFE catheter was inserted in the left antecubital vein 30 min before the blood drawing. Blood was collected in chilled heparinized tubes and centrifuged immediately (1000 *g*, 10

min). The influence of dietary components was tested in blood samples collected at 10 a.m. and 3:30 p.m. (before and after a meal).

Sample preparation

Extraction of free methoxylated amines. A 2-ml volume of plasma mixed with 3.2 pmol of the internal standard MHBA were poured into 400 μl of 2 *M* perchloric acid in an ice-cold tube and centrifuged at 8800 *g* for 10 min. The supernatant was adjusted to pH 7 with 5 *M* ammonia and made up to 6 ml with GBW. The mixture was applied to a Dowex 50 W X2, H^+, column (50 mm × 5 mm I.D.). The column was then washed with 2 ml GBW, and the amines were eluted with 2 *M* ammonia containing 200 ml/l methanol. The first 0.5-ml portion was discarded, and the amines were collected in the subsequent 1.5 ml. The Dowex eluate was evaporated to dryness under vacuum at 40°C. The residue, redissolved in 100 μl of 0.01 *M* HCl, was used for HPLC.

Assay of total (free + conjugated) NMN and MN. Acid hydrolysis was carried out as follows. The supernatant of 1 ml deproteinized plasma (see above) including the internal standard (10 pmol of MHBA) was poured into a glass tube containing 5 ml of 0.1 *M* perchloric acid, previously heated to 95°C in a dry hot bath. The hydrolysis of conjugated methoxylated amines lasted 10 min at 95°C and was then immediately stopped in an ice-bath. The hydrolysed sample was adjusted to pH 7 with 200 μl of 5 *M* NH_4OH, and the deconjugated methoxylated amines were extracted as described above for free amines.

Additional purification of the Dowex eluate. Occasionally, interferences were observed in the HPLC chromatogram of the plasma from drug-treated patients. The evaporated Dowex eluate, redissolved in HCl as above, was made up to 1 ml with GBW and further purified on an Amberlite CG 50 column (70 mm × 5 mm I.D.). The column was washed with 10 ml of GBW and 0.8 ml of 0.2 *M* NH_4OH, and elution was performed with 1.2 ml of 0.2 *M* NH_4OH. The ammoniacal eluate was evaporated to dryness under vacuum at 40°C, dissolved in 100 μl of 0.01 *M* HCl, and used for HPLC assay.

HPLC analysis

A 40-μl volume of the Dowex eluate was usually injected into the chromatograph. The working potentials were +550 mV (V1) and +780 mV (V2) for the two electrodes. The recorded signal given by the first working electrode was substracted from the signal of the second working electrode. The sensitivity was set at 0.5 nA for both electrodes, and the flow-rate was 1.0 ml/min. The retention times for NMN, MN and MHBA were 6.75, 9.60 and 15.6 min, respectively. A typical chromatogram of the plasma from a healthy human subject is shown in Fig. 1.

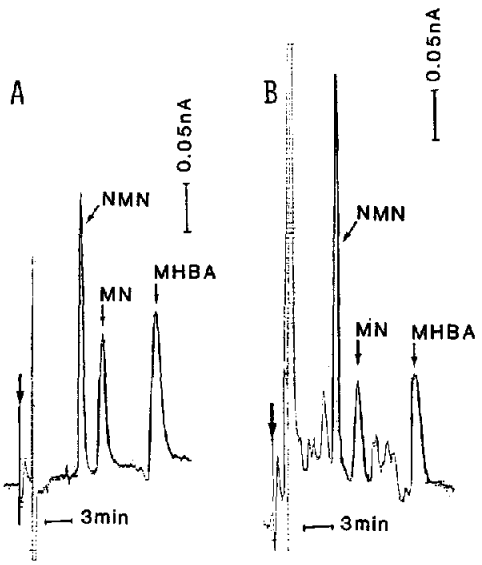

Fig. 1. Typical chromatograms of (A) authentic methoxyamines NMN and MN (2 pmol) and MHBA (4 pmol), and (B) hydrolysed plasma from a healthy human subject. Dowex eluate was evaporated and reconstituted with 100 μl of 0.01 *M* HCl, and 40 μl were injected. Original plasma concentrations were 7.2 pmol/ml for NMN and 3.7 pmol/ml for MN. Column, Hypersil ODS C_{18} 5 μm, 100 m × 4.6 mm I.D.; eluent, 50 m*M* monosodium phosphate, 50 m*M* trisodium citrate, 2 m*M* sodium octanesulphonate, 2 m*M* disodium EDTA and 100 ml/l methanol, final pH, 3.5 with 5 *M* HCl; flow-rate, 1.0 ml/min; working potential, −550 and +780 mV; sensitivity, 0.5 nA.

RESULTS

Development of the method

Hydrolysis of conjugated methoxylated amines. A 1-ml volume of human plasma was deproteinized and mixed with 5 ml of hot perchloric acid (95°C) at different molarities to produce pH values ranging from 0.8 to 2.0. The mixtures were heated at 95°C for 10, 20 or 30 min. The highest levels of methoxylated amines were obtained when the hydrolysis was conducted at pH 1 for 10 min (Fig. 2). In agreement with Foti *et al.* [3] we observed that the hydrolysis was less efficient at pH values higher than 1.4. On the other hand, pH values less than 1.0 or heating times longer than 10 min resulted in amine loss, especially for MN. The stability of the internal standard MHBA to the hydrolysis conditions was evaluated by processing deproteinized plasma samples spiked with 3.2 pmol of authentic MHBA throughout the whole procedure (hydrolysis + Dowex procedure). When heated for 10–30 min, MHBA was stable in the pH range 1.0–2.0 but, like NMN and MN, it was affected at pH values below 1.0.

Dowex 50 procedure. To investigate the features of the Dowex extraction, 1 ml of saline sample containing 3.2 pmol of the three authentic amines (NMN, MN

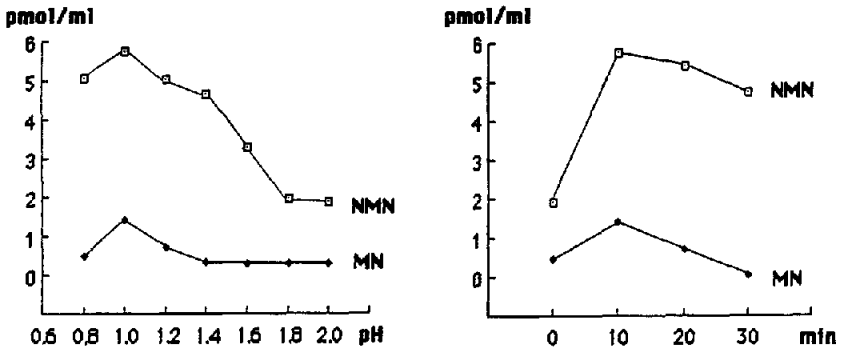

Fig. 2. Effect of pH and time on the deconjugation of plasma-conjugated NMN and MN submitted to acid hydrolysis at 95°C.

and MHBA) were processed throughout the Dowex procedure in various conditions (Fig. 3).

The optimal pH value for metanephrine exchange on Dowex 50 W X2 (obtained with 5 *M* NH_4OH [9]) was pH 7. The recoveries were greater than 90% for NMN, and near 80% for MN and MHBA.

From literature data [3,8], ammoniacal methanol was chosen to elute the metanephrines from Dowex 50 W X2, H^+. We obtained the greatest recoveries with 2 *M* NH_4OH in 20% methanol. In routine analysis, the first 0.5-ml portion

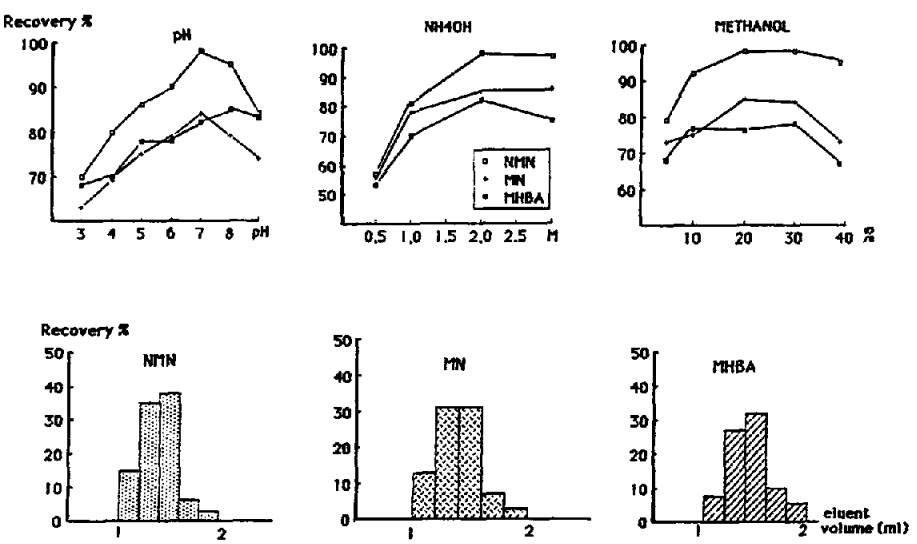

Fig. 3. Main features of the Dowex 50 procedure. (Upper panels) effect of pH, NH_4OH molarity in ammonia–methanol (80:20, v/v), and methanol concentration in 2 *M* NH_4OH eluent. (Lower panels) elution diagrams of authentic NMN, MN and MHBA from Dowex under the final conditions, pH adjusted to 7, elution with 2 *M* NH_4OH–methanol (80:20, v/v).

was discarded and the subsequent 1.5 ml of eluate was saved. The recoveries of authentic exogenous compounds were 98.0 ± 3.6, 84.5 ± 3.6 and 81.9 ± 3.6% (mean ± S.D., $n = 9$) for NMN, MN and MHBA, respectively.

Additional purification of Dowex eluate. A 1-ml saline sample containing 3.2 pmol of authentic NMN, MN and MHBA was first purified on the Dowex 50 column. The evaporated eluate, reconstituted in 1 ml of 0.01 *M* HCl, was then applied, after appropriate pH adjustment, to a Sephadex G-10 or an Amberlite CG 50, NH_4^+, column.

We used a Sephadex G-10 column (70 mm × 5 mm I.D.) to purify urinary metanephrines as described by Westerink and Tenkate [10]. Under the final conditions, the mean recoveries (± S.D., $n = 9$) of the three methoxylated amines through the whole procedure (Dowex 50 followed by Sephadex G-10) were 77.7 ± 4.2, 80.7 ± 5.4 and 78.8 ± 5.4% for NMN, MN and MHBA, respectively.

Amberlite CG 50 in the NH_4^+ form has been used to extract metanephrines from Dowex 50 eluates [9,12]. We found 0.2 *M* NH_4OH to be suitable for elution. Increasing the molarity to above 0.2 *M* enhanced the elution of interfering compounds while only slightly improving the recovery of methoxylated amines. For routine analysis, the Dowex eluate was evaporated to dryness, redissolved in 100 µl of 0.01 *M* HCl and made up to 1 ml with GBW. This solution was applied to the Amberlite column (70 mm × 5 mm I.D.) at pH 7, and processed as described in *Sample preparation*. Under the final conditions, the mean recoveries (± S.D., $n = 8$) through the whole procedure (Dowex 50 followed by Amberlite CG 50) were 80.0 ± 7.9, 74.2 ± 4.0 and 78.3 ± 4.0% for NMN, MN and MHBA, respectively.

HPLC conditions. Because the metanephrines are strongly retained with the mobile phase routinely used in catecholamine assays, several authors have introduced HPLC systems containing stronger eluting agents (acetonitrile, trichloroacetic acid, perchloric acid) [8,11,13]. Since mixtures are highly corrosive for HPLC materials, we preferred a citrate–phosphate buffer containing octane sulphonate and methanol. Increasing octane sulphonate and decreasing citrate concentrations both greatly increased the retention times of NMN, MN and MHBA (Fig. 4). Increasing methanol concentration or pH value had a minor effect on the retention times (Fig. 4), but greatly augmented the peak heights, either by sharpening the peaks (methanol) or by favouring phenol oxidation (pH). Adequate chromatographic resolution, together with a reasonable analysis time, was obtained with a mobile phase containing 50 m*M* citrate, 50 m*M* phosphate, 2 m*M* octane sulphonate and 100 ml/l methanol. To minimize the interference of synephrine on the NMN peak, the pH of the mobile phase was adjusted to 3.5 (see below).

The assumed NMN and MN peaks found in plasma extracts were tentatively identified on the basis of retention behaviour with different mobile phases and co-chromatography with reference compounds. Further identification was achieved by varying the oxidation potential (V2) of the detector for the plasma

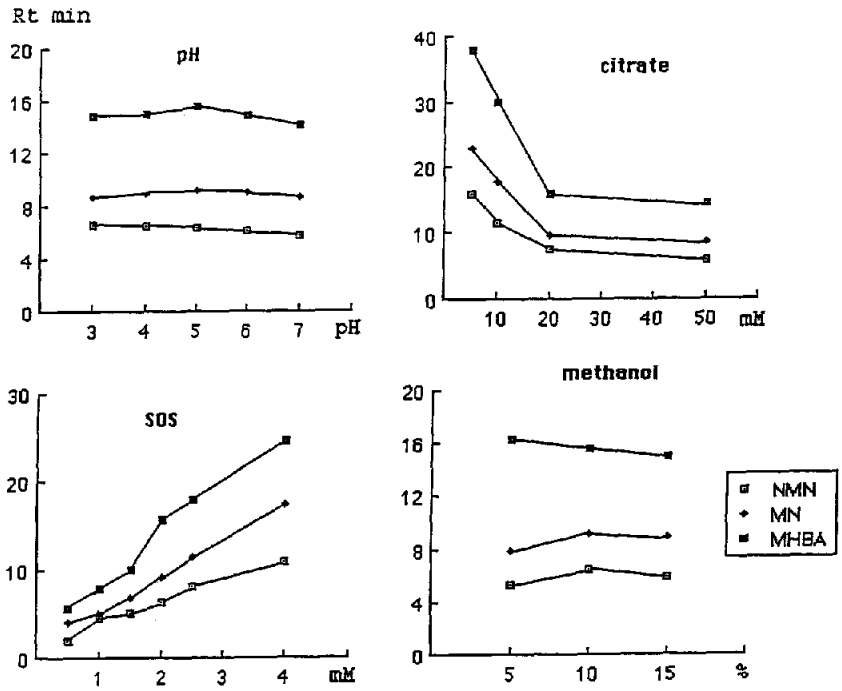

Fig. 4. Effect of the mobile phase composition and pH on the retention times of methoxylated amines. The mobile phase consisted of 50 m*M* monosodium phosphate, 2 m*M* disodium EDTA mixed with trisodium citrate, octanesulphonate and methanol at various concentrations.

extract and reference compounds. In both methods, the assumed NMN and MN peaks from plasma behaved as the authentic amines (Fig. 5).

Evaluation of the final method

Linearity and sensitivity. The detection limits calculated from the peak height equivalent to 2% of full scale were 0.10, 0.12 and 0.12 pmol for NMN, MN and MHBA, respectively. The detection was linear up to 60 pmol (amount injected).

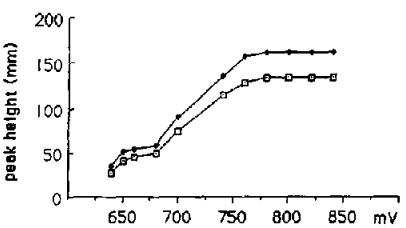

Fig. 5. Relationship between peak heights and the oxidizing potential (voltammogram) for (□) authentic NMN and (◆) the assumed NMN peak in the plasma of a healthy adult.

TABLE II

PRECISION OF THE METHOD (MEAN ± S.D.)

Compound	Intra-assay (n = 30)		Inter-assay (n = 15)	
	pmol/ml	C.V. (%)	pmol/ml	C.V. (%)
NMN free	2.33 ± 0.33	14.2	2.41 ± 0.31	12.9
NMN total	7.12 ± 1.04	14.6	6.96 ± 0.93	13.4
MN free	0.96 ± 0.16	16.7	0.96 ± 0.31	32.3
MN total	2.49 ± 0.38	15.3	2.48 ± 0.35	14.1

Precision. Day-to-day precision was determined from analysis of a plasma pool from hypertensive patients, over a period of fifteen days. Within-batch precision was evaluated by assaying the plasma pool thirty consecutive times. The data in Table II show variations of less than 15% for intra-assay precision; the between-run precision was *ca.* 15% for all compounds except free MN, whose values were very low.

Recoveries in plasma. Known concentrations of each compound were added to aliquots of plasma pools from healthy subjects. The amount measured in the final extract sample of each supplemented sample, minus the value of the compound in the plasma pool, was compared with the known amount added. The recoveries thus obtained were 97.7 ± 2.3 and 83.7 ± 2.9% (mean ± S.D., n = 14) for NMN and MN, respectively.

Specificity. Table I compares the relative retention times and peak heights of the two metanephrines with those of several possible interfering compounds.

TABLE III

FREE AND TOTAL METHOXYLATED AMINES IN PLASMA OF ADULT HEALTHY SUBJECTS AT REST

Values are pmol/ml, mean ± S.D.; no sex-related differences in statistical test (U-Mann and Whitney).

	Men (n = 6)	Women (n = 8)	Both sexes (n = 14)
NMN			
Free	0.57 ± 0.34	0.28 ± 0.17	0.41 ± 0.30
Conjugated	4.20 ± 0.69	4.43 ± 1.54	4.33 ± 1.27
Conjugated (%)	85.0 ± 10.8	93.0 ± 5.09	89.5 ± 8.61
MN			
Free	0.14 ± 0.07	0.16 ± 0.08	0.15 ± 0.07
Conjugated	2.20 ± 0.66	2.20 ± 0.48	2.21 ± 0.52
Conjugated (%)	92.9 ± 4.41	91.7 ± 5.37	92.2 ± 4.86

Equimolar amounts of each compound were injected using the mobile phase described in *Chromatographic conditions*, adjusted to pH 3.5 or 5.5 with 5 *M* HCl. The interference study was conducted under two sets of conditions: (i) mobile phase pH 5.5 or 3.5 with a single working electrode at +780 mV; (ii) mobile phase pH 3.5 with the double working electrode system +550 mV (V1) and +780 mV (V2).

With the mobile phase pH 5.5 and a single potential (+780 mV) the following compounds exhibited measurable peaks with relative retention times in the range of metanephrines (0.9–1.7): DHBA, synephrine, dopamine and isoferulic acid (Table I). Of these, only synephrine, an endogenous sympathomimetic amine, was quantitatively extracted by the Dowex 50 procedure and could interfere with the plasma NMN assay. This interference was greatly reduced by using a mobile phase at pH 3.5. On the other hand, using the double electrode working system further improved the assay specificity by reducing the peaks of most compounds, some of which (*i.e.* catecholamines) could interfere if present in large amounts (patients with pheochromocytomas).

Tyramine, which is extracted by the Dowex 50 column, exhibited a significant peak under all conditions at a relative retention time far enough from that of the internal standard MHBA to allow efficient separation. If necessary for samples with large amounts of tyramine, the relative interference of the tyramine peak can be reduced to 0.5% of NMN by lowering the potential (V2) to +750 mV.

No detectable peak was found under any of the above conditions with a number of antihypertensive drugs (Table I) and the following endogenous or exogenous compounds: homogentisic acid, *m*- or *p*-hydroxymandelic acid, *m*- or *p*-methoxyphenylacetic acid, *o*- or *m*-methoxymandelic acid, homoveratric acid and homoveratrylamine. Acetylsalicylic acid and paracetamol (acetaminophen) exhibited very low relative retention times and did not interfere in the metanephrine assay (Table I).

Normal values in healthy adults

NMN and MN concentrations in plasma collected from fourteen healthy subjects in the supine position are shown in Table III. The major part (85–93%) of plasma methoxyamines was in the conjugated form. No sex-related differences were observed for free and total NMN and MN. No interfering effect of dietary components was observed in plasma samples taken after a meal.

DISCUSSION

We have developed a method for the assay of free and conjugated metanephrines in plasma. Only two authors have previously studied free and conjugated plasma NMN [3,14], but no data are available for conjugated MN. Our data show that, like NMN, 90% of circulating MN is conjugated in human plasma (Table III). Optimal deconjugation of conjugated NMN was achieved in 10 min

at pH 1.0. However, we found perchloric acid to be more convenient than trichloroacetic acid for plasma deproteinization and acidification prior to hydrolysis. The finding that conjugated metanephrines are readily hydrolysed at acid pH, confirms that the major conjugates of plasma NMN and MN are sulphates [3,14].

From the available literature on metanephrine assay in urine [9,12,15,16] or plasma [17], Dowex 50 W X2, H^+, was chosen to extract metanephrines from plasma. AGMP 50, previously used for urine samples [8], was also examined as an alternative to Dowex 50 W X2 to purify the plasma sample. The details (fixation pH, composition of ammoniacal methanol as eluting agent and elution diagram) of the extraction of 3-O-methylated amines using AGMP 50 were very similar to those using Dowex 50 W. We chose Dowex 50 for metanephrine assay because of its current availability in laboratories and lower cost than AGMP 50.

In our hands, extrapolation to plasma of the organic solvent procedure described by Buu *et al.* [13] to extract metanephrines from urine led to unsatisfactory HPLC profiles and poor recovery of amines. In agreement with previous findings [9,16] one essential condition for quantitative recovery of the 3-O-methoxylated amines through Dowex 50 W X2 was a low Na^+ content in the sample. Therefore prior to the Dowex extraction, the pH was adjusted with ammonium hydroxide, and the sample was diluted six-fold. In line with Foti *et al.* [3] we found that ammoniacal methanol is more suitable than aqueous ammonia for elution of 3-O-methoxylated amines from Dowex 50. Indeed, adding methanol to the ammoniacal eluent allowed a reduction of the elution volume and decreased the risk of interfering compounds, while shortening the evaporation time. Just 2 ml of 2 *M* NH_4OH in 20% methanol were sufficient to elute 80–95% of the methoxylated amines fixed on the Dowex 50. Like previous authors [13,18,19] we used MHBA as an internal standard: it has chemical features very similar to those of NMN and MN and was stable to the hydrolysis procedure.

The Dowex 50 eluate can be used for direct HPLC analysis of plasma methoxylated amines. However, interferences were noted with plasma samples from some drug-treated hypertensive patients. Two additional purifying systems were developed: Sephadex G-10 and Amberlite CG 50. Similar recoveries were obtained with both procedures. Each of these systems has advantages and disadvantages. Sephadex G-10 columns are easy to prepare and can be reused several times, but the sample filtration takes *ca.* 1 h. The preparation time for the Amberlite CG 50 resin in the NH_4^+ form is quite long, and the columns must be discarded after a single assay. However, the extraction step is simple and rapid. Occasionally, when interferences were present in the HPLC profile, the double-step purification was highly convenient for HPLC assay; however, this was not usually necessary.

In previous methods for NMN assay in urine, electrochemical detection of NMN has been generally performed at a working potential ranging from +800 to +1000 mV [13,15,16,19,20]. However, such high potentials favour oxidation of several compounds, including monophenols, and increase the number of pos-

sible interferences. Since NMN is conveniently oxidized at a potential in the range +750 to +780 mV, we used a potential of +780 mV to minimize the interference of synephrine (Fig. 6). Furthermore, as shown in Table I, the dual-series electrode arrangement greatly improves the assay specificity.

Interferences of antihypertensive agents (β- or α-blockers, α-methyldopa) on urinary methoxyamines were reported when these amines were assayed by fluorimetric or spectrophotometric methods [21–24]. No similar data are available for plasma methoxyamines. In our HPLC method, no interferences were observed with nine antihypertensive agents (Table I), either because they gave no peak (adrenergic blockers, captopril, clonidine) or because the peak was not in the range of NMN or was totally eliminated by the double working electrode system (α-methyldopa) (Table I). An interference of acetylsalicylic acid was previously reported [25,26] in the urinary methoxyamine assay with the colorimetric method of Pisano. In our HPLC method, this compound exhibited a very small peak at a relative retention time of 0.48 *versus* NMN (Table I). Paracetamol (acetaminophen), whose interference in the HPLC assay of urinary metanephrine has been previously reported [27], did give a peak in our system but its interference was not crucial because of the low relative retention time (Table I).

In addition to metanephrines, substantial amounts of the sympathomimetic amines (synephrine, octopamine and tyramine) are present in human plasma and urine. Synephrine and octopamine are thought to be stored in the sympathic nerve endings and are released with nerve discharge and compete with norepinephrine for postsynaptic receptors [17,26]. Tyramine has both endogenous and exogenous origins, as a metabolite of tyrosine or/and as a component of several foods (cheese, white wine, bananas, oranges, avocados) [28]. As previously reported [8,29], these three amines are extracted through the Dowex 50 column and give peaks with electrochemical detection. In our HPLC method, the retention times of octopamine and tyramine are sufficiently different from those of NMN and MHBA, respectively, to avoid interference. The presence of synephrine is more inconvenient since its peak is very near to that of NMN and its amount in normal subjects is similar to that of NMN. In our study, great care was taken to reduce the interference of this amine by using an acid mobile phase (Table I) and

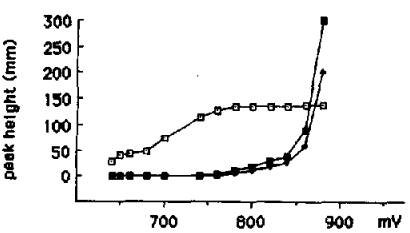

Fig. 6. Voltammogram for (□) authentic NMN, (◆) authentic synephrine and (■) plasma spiked with synephrine.

setting the working potential V2 at no more than +780 mV, because synephrine is readily oxidized at higher potentials (Fig. 6).

CONCLUSION

We have developed an HPLC method for the assay of free and total methoxylated amines in plasma. The principal advantages of the method stem from the high sensitivity and specificity for the substances of interest. Plasma NMN and MN assay will be useful for diagnosis in patients with hypertensive pathology.

ACKNOWLEDGEMENT

We acknowledge the amiable and skillful assistance of Rose Marie Cottet-Emard.

REFERENCES

1 N. D. Vlachakis and V. DeQuattro, *Biochem. Med.*, 20 (1977) 107.
2 I. J. Kopin, R. J. Polinsky, J. A. Oliver, I. R. Oddershede and M. H. Ebert, *J. Clin. Endocrinol. Metabol.*, 57 (1983) 632.
3 A. Foti, M. Adachi and V. DeQuattro, *J. Clin. Endocrinol. Metab.*, 55 (1982) 81.
4 S. Yoneda, N. Alexander, N. D. Vlachakis and R. F. Maronde, *Am. J. Physiol.*, 247 (1984) 208.
5 K. Iinuma, I. Ikeda, M. Takai, K. Kurata, T. Ogihara, Y. Kumuhara, H. Shionoiri and Y. Kaneko, *Nippon Naibumpi Gakkai Zasshi*, 59 (1983) 1667.
6 B. H. C. Westerink and T. B. A. Mulder, *J. Neurochem.*, 36 (1981) 1449.
7 L. Peyrin, *Rev. Fr. Etud. Clin. Biol.*, 13 (1968) 88.
8 J. G. Flood and R. B. McComb, *Clin. Chem.*, 27 (1981) 1268.
9 L. Peyrin and R. Mornex, *Pathol. Biol.*, 16 (1968) 447.
10 B. H. C. Westerink and N. Tenkate, *J. Clin. Chem. Clin. Biochem.*, 24 (1986) 513.
11 N. G. Abeling, A. H. van Gennip, H. Overmars and P. A. Voute, *Clin. Chim. Acta*, 137 (1984) 211.
12 K. Taniguchi, Y. Kakimoto and M. D. Armstrong, *J. Lab. Clin. Med.*, 64 (1964) 169.
13 N. T. Buu, M. Angers, D. Chevalier and O. Kuchel, *J. Lab. Clin. Med.*, 104 (1984) 425.
14 S. Yoneda, N. Alexander and N. D. Vlachakis, *Biochem. Pharmacol.*, 33 (1984) 2029.
15 L. M. Bertrani-Dziedzic, A. M. Krstulovic, S. W. Dziedzic, S. G. Gitlow and S. Cerqueira, *Clin. Chim. Acta*, 110 (1981) 1.
16 J. Jouve, N. Mariotte, C. Sureau and J. P. Muh, *J. Chromatogr.*, 274 (1983) 53.
17 K. Kobayashi, V. DeQuattro, J. Bornheimer, R. Kolloch and L. Miano, *Life Sci.*, 26 (1970) 1821.
18 P. J. Orsulak, P. Kizuka, E. Grab and J. J. Schildkraut, *Clin. Chem.*, 29 (1983) 305.
19 R. E. Shoup and P. T. Kissinger, *Clin. Chem.*, 23 (1977) 1268.
20 N. C. Parker, C. B. Levtzow, P. W. Wright, L. L. Woodward and J. F. Chapman, *Clin. Chem.*, 32 (1986) 1473.
21 L. Miano, R. Kolloch and V. DeQuattro, *Clin. Chim. Acta*, 95 (1979) 211.
22 G. L. Munion, J. F. Seaton and T. S. Harrison, *J. Surg. Res.*, 35 (1983) 507.
23 D. J. Savory, *Ann. Clin. Biochem.*, 21 (1984) 446.
24 P. Yates and C. Weinkove, *Ann. Clin. Biochem.* 23 (1986) 487.
25 B. Spilker, B. S. Watson and J. W. Woods, *Ann. Clin. Lab. Sci.*, 13 (1983) 16.
26 C. D. Williams, M. W. Couch, M. Thonoor and J. M. Midgley, *J. Pharm. Pharmacol.*, 39 (1987) 153.
27 S. P. Wilson, D. L. Kamin and J. M. Feldman, *Clin. Chem.*, 31 (1985) 1093.
28 S. Udenfriend, *Arch. Biochem. Biophys.*, 85 (1959) 487.
29 G. P. Jackman, *Clin. Chim. Acta*, 120 (1982) 137.