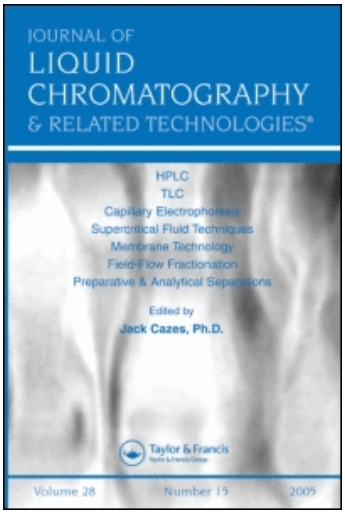


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# USE OF ETHOXY-HOMOLOGS AS INTERNAL STANDARDS FOR DETERMINATION OF URINARY VANILLYLMANDELIC ACID AND NORMETANEPHRINE IN MAN BY HIGH PERFORMANCE LIQUID CHROMATOGRAPHY

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ABSTRACT

Synthesis of the ethoxy-homologs of vanillylmandelic acid (3-ethoxy-4-hydroxymandelic acid, EMA) and normetanephrine (3-ethoxy-4-hydroxyphenylethanolamine, EHPEA) and their use in minor modifications of existing assays on human urine are described. For analysis of vanillylmandelic acid, the homologous internal standard is added to an aliquot of urine, organic acids are then extracted into ethyl acetate and back extracted into 10% $K_2CO_3$, and finally extracted a second time into ethyl acetate. After evaporation of the ethyl acetate, the residue is redissolved in the mobile phase to be used in the chromatography, and injected onto a reverse phase column. Comparison of results with a gas



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chromatography-mass spectrometry (GCMS) assay gave almost identical results (96% ± 2%, mean ± SE, n=10). Analysis of normetanephrine is performed by addition of internal standard to an aliquot of urine before heat hydrolysis of amine conjugates. The amines are adsorbed onto columns of Bio Rex 70, and then eluted with a solution of 1 M $NH_4OH$, which is then evaporated to dryness. After residue is redissolved in borate buffer, the amines are extracted into toluene:isoamyl alcohol and back extracted into 0.1 M acetic acid before injection onto a reverse phase column. Correlation with a GCMS assay gave similar results (102% ± 4%, mean ± SE, n=9).

## INTRODUCTION

The principal pathways for metabolism of norepinephrine (NE) include O-methylation and deamination. The major urinary metabolites of NE are therefore 4-hydroxy-3-methoxyphenylethylene glycol (MHPG), 4-hydroxy-3-methoxymandelic acid (vanillylmandelic acid, VMA) and 4-hydroxy-3-methoxyphenylethanolamine (normetanephrine, NMN). Studies of sympathetic nerve function in psychiatric and neurological disorders have made accurate, convenient measurement of these three O-methylated metabolites of NE important.



We recently reported a reverse phase high performance liquid chromatographic (HPLC) method for determining levels of MHPG in human urine, using 3-ethoxy-4-hydroxyphenylethylene glycol as internal standard (1). Synthesis of this compound has been previously described (2).

Urinary VMA has previously been assayed by GCMS (3) and by HPLC with UV detectors (4,5), or electrochemical detectors (6,7,8,9). Normetanephrine has also been determined by spectrophotometry (10), by GCMS (11), and by HPLC, either after conversion to vanillin (12) or directly by fluorescence (13) or electrochemical detectors (14,15). Although deuterated internal standards are available for GCMS techniques, suitable internal standards for HPLC methods have not been described. In most methods, either no internal standard is used or tritiated

standards, which require separate assay by scintillation spectrometry for determination of recovery are used. Although 4-hydroxymandelic acid (POMA) has been used as internal standard for determination of VMA by UV detection (4), its use is complicated by its normal occurrence in human urine. It also lacks a catechol structure, oxidation of which is the basis for detection of the O-methylated metabolites of norepinephrine (16). Similarly, 4-hydroxy-3-methoxybenzylamine has been used as internal standard for normetanephrine measurement (14), but it is poorly separated from other peaks.

## MATERIALS AND METHODS



### Apparatus

A model 6000A solvent delivery system and model U6K Universal Injector, both from Waters Associates (Milford, Mass., USA) were used. The electrochemical detector from Bioanalytical Systems (West Lafayette, Ind., USA) consisted of an LC-4A controller and a TL-5 cell (glassy carbon electrode and Ag/AgCl reference electrode). Analytical columns (micro Bondapak C18 stainless steel reverse phase, 3.9 mm x 30 cm, 10 micron particles) and guard columns (packed with Bondapak C18/Corasil) were obtained from Waters Associates (Milford, Mass., USA). Peaks were recorded on an Omniscribe recorder from Houston Instruments (Austin, Texas, USA). The Speed Vac Concentrator was obtained from Savant Instrument Company (Hicksville, NY, USA) and the Dribath from Thermolyne Corp. (Dubuque, Iowa, USA).

### Chromatographic Conditions

Urinary NMN was determined by isocratic elution mode of reverse phase HPLC. A step gradient was used in measurement of urinary VMA by using the solvent selection knob on the pump to switch from a lower to a higher concentration of acetonitrile just as the sample was injected. The system was returned to 2% acetonitrile after sufficient time at the higher concentration to

cause elution of late peaks. Since the effects of a switch are delayed about 15 minutes, this has the effect of eluting the VMA and EMA peaks at the lower concentration. The eluents for VMA contained 0.1 M phosphoric acid, 0.27 mM disodium EDTA, and either 2% or 6% acetonitrile in distilled water, adjusted to pH 3.1 with 10 N NaOH. The eluents were filtered through 0.45 micron Millipore filters before addition of acetonitrile. Each morning the eluents were degassed by placing each bottle with two stirring bars on a magnetic stirring plate under vacuum. The eluent for NMN was identical to those used for VMA except that it contained 2 mM heptanesulfonic acid (Fisher Scientific, Fair Lawn, NJ, USA), added before filtering the eluent, and 5% acetonitrile. The electrochemical detector was operated at 0.85v vs. Ag/AgCl. Flow rate was 1 ml/min., and temperature was ambient.



## Reagents

For standards, VMA (D,L-4-hydroxy-3-methoxymandelic acid, Sigma Chemical Company, St. Louis, MO, USA) and normetanephrine (DL-Normetanephrine HCl, Sigma Chemical Company, St. Louis, MO, USA) were used. Acetonitrile, ethyl acetate, toluene, and phosphoric acid (85%) were HPLC grade from Fisher Scientific (Fair Lawn, NJ, USA). Bio Rex 70, 50-100 mesh, sodium form, was obtained from Bio Rad (Richmond, CA, USA). All other chemicals were reagent grade. Water was distilled in glass.

## Synthesis of Internal Standards

3-Ethoxy-4-hydroxymandelic acid: A solution of ethyl 4-benzyloxy-3-ethoxymandelate synthesized as previously described (2), in 10 ml of 0.9 N NaOH and 5 ml of ethanol was stirred overnight. Acidification and extractive workup gave 564 mg (62%) of 4-benzyloxy-3-ethoxymandelic acid, m.p. 90-92°C (recrystallized from ethyl acetate). Analysis calculated for $C_{17}H_{18}O_5$: C, 67.53; H, 6.00. Found: C, 67.81; H, 6.13. Hydrogenolysis of this compound (390 mg, 1.29 mmole) over Pd-C in ethanol gave 258 mg (95%) of 3-ethoxy-4-hydroxymandelic acid, m.p. 119-121°C

(recrystallized from ethyl acetate/petroleum ether.) Analysis calculated for $C_{10}H_{12}O_5$: C, 56.60; H, 5.70. Found: C, 56.89; H, 5.91. NMR (220 MHz, $CD_3Cl$, 10% $CD_3OD$): 1.58 (t, J=7 Hz, 3H, $C\underline{H}_3CH_2^-$); 4.26 (q, J=7 Hz, 2H, $CH_3\underline{C}H_2^-$); 5.20 (s, 1H, $^-C\underline{H}$,$OH^-$); 6.90-7.10 (m, 3H, aromatic).

3-Ethoxy-4-hydroxyphenylethanolamine: Lithium aluminum hydride reduction of 4-benzyloxy-3-ethoxybenzaldehyde trimethylsilylcyanohydrin, prepared from 1 g of 4-benzyloxy-3-ethoxybenzaldehyde in the usual manner (2), gave 887 mg (79%) of 4-benzyloxy-3-ethoxyphenylethanolamine, m.p. 95-96°C (recrystallized from cyclohexane/ethyl acetate). Analysis calculated for $C_{17}H_{21}NO_3$: C, 71.05; H, 7.37; N, 4.90. Found: C, 70.63; H, 7.69; N, 4.73. A 550 mg sample of this compound was converted to the oxalate salt and was hydrogenolyzed over 10% Pd-C in methanol to give 212 mg (46%) of 3-ethoxy-4-hydroxyphenylethanolamine as the neutral oxalate, m.p. 200-203°C (recrystallized from 95% ethanol). Analysis calculated for $C_{10}H_{15}NO_3 \cdot \frac{1}{2}\ H_2C_2O_4$: C, 54.53; H, 6.66; N, 5.78. Found: C,54.29; H,6.65; N, 5.57. NMR (220 MHz, $D_2O$): 1.40 (t, J=7 Hz, 3H, $C\underline{H}_3CH_2^-$); 3.25 (m, 2H, $^-CHC\underline{H}_2NH_2$); 4.16 (q, J=7 Hz, 2H, $CH_3\underline{C}H_2^-$); 4.90 (m, 1H,$^-C\underline{H}OHCH_2^-$); 6.91-7.08 (m, 3H, aromatic).

Procedure for Determination of Urinary VMA

To one ml of urine was added 50 microliters of a solution containing 200 micrograms/ml of EMA either alone or in combination with 100 micrograms/ml of VMA. Four hundred mg NaCl were then added, and the mixture was adjusted to pH 1 with 12 N HCl. VMA was then extracted three times using 1 ml of ethyl acetate, and the extracts pooled. VMA was then back-extracted into 1 ml of 10% $K_2CO_3$ (pH 12), and 400 mg NaCl added to the recovered aqueous phase, which was then adjusted to pH 1 with 12 N HCl. VMA was again extracted three times using 1 ml of ethyl acetate. The pooled organic phase was evaporated to dryness in a Speed-Vac Concentrator, and the residue redissolved in 1 ml of mobile phase

before injection onto a reverse phase HPLC column. Duplicate urine samples were assayed by GCMS using the method of Takahashi (17).

Procedure for Determination of Urinary NMN

Samples were analyzed by a modification of the method of Jackman (13). To one ml of urine was added 50 microliters of a solution containing 20 micrograms/ml of EHPEA either alone or in combination with 10 micrograms/ml of NMN. The sample was adjusted to pH 1 with 12 N HCl, then heated in a Dribath at 90°C for 30 minutes. After cooling to room temperature, the sample was adjusted to pH 6.5 with NaOH, then applied to a column of Bio Rex 70, pH 6.5 in 0.02 M phosphate buffer. The ion exchange resin was prepared by washing the resin several times with distilled water until the supernatant was clear, then resuspending the resin in 0.02 M phosphate buffer and adjusting to pH 6.5. Columns were prepared by adding the slurry to a glass column with a 50 ml reservoir and 6 mm inside diameter, over a small glass wool plug, to a height of 46 mm (resin volume of 1.3 ml). The sample was first added to the column and the effluent discarded. The column was then washed with 10 ml of 0.02 M phosphate buffer (pH 6.5), followed by 3 ml of distilled water and 1 ml of 1 M $NH_4OH$, all effluents being discarded. A subsequent 2.5 ml wash of 1 M $NH_4OH$ was then collected, and evaporated to dryness in a Speed-Vac Concentrator. The residue was redissolved in 0.1 ml of 0.5 M borate buffer (pH 9.0). NMN was extracted into 4 ml of toluene:isoamyl alcohol (3:2), then back-extracted into 0.5 ml of 0.1 M acetic acid before injection onto a reverse phase HPLC column. Duplicate urine samples were assayed by GCMS using a method previously described (18).

Detection Limits and Linearity of Response

The detection limit for VMA was approximately 0.3 nanograms at 10 nanoamps full scale sensitivity, and for NMN was approximately 0.2 nanograms at a sensitivity of 5 nanoamps full

scale. Linearity of response was examined by injecting 50 microliters of seven different dilutions of standard solutions containing each of the metabolites and each of the internal standards. Correlation of peak height with amount injected revealed linear relationships for all four compounds (r values were 0.9986, 0.9991, 0.9995, and 0.9995 for VMA, EMA, NMN, and EHPEA respectively) over a range of 0.5-10 ng for the metabolites and 1-20 ng for the internal standards. A linear response was only obtained if all injections were of the same volume.

Peak Identification and Detector Response

Peaks on chromatograms of urine were identified as either VMA or NMN by comparing retention times and voltage curves to those of standard solutions of these metabolites. Absence of peaks interfering with the internal standards was confirmed by analysis of paired samples, one of which had no internal standard added. The greater molecular weights of the internal standards than their respective metabolites increases their retention times sufficiently to consistently separate the compounds on chromatography, but not so much as to make isocratic elution impractical. The voltage curves of both of the internal standards were very similar to those of their respective metabolites (Figs. 1 and 2). A detector voltage of +0.85v was used in both assays, since this resulted in adequate peak heights, while avoiding the unstable baseline which occurs with higher voltages.



## RESULTS AND DISCUSSION

### Method Development

VMA: Most methods reported for analysis of urinary VMA have used extraction into ethyl acetate from acidified urine (5,6,7). Presence of interfering peaks has led to modifications of this basic procedure, including either an initial extraction of contaminants from alkalinized urine into ethyl acetate (4), or a subsequent extraction from ethyl acetate into TRIS buffer,

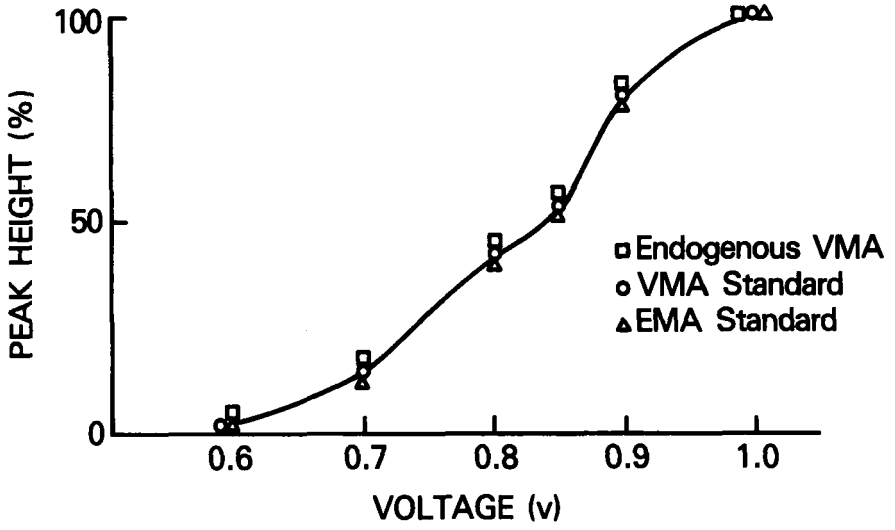


FIGURE 1. Relative Detector Response as a Function of Oxidizing Potential vs. Ag/AgCl Electrode for Endogenous VMA, VMA Standard and EMA Standard



followed by small column chromatography on diethylaminoethyl cellulose (8). A second extraction from ethyl acetate into an alkaline aqueous solution results in sufficient removal of contaminating peaks so that further cleanup is not required. A repeat extraction into ethyl acetate is useful because alkaline samples cannot be injected, and because it facilitates concentration of the sample by evaporation. EMA is a useful internal standard because it is consistently separated from VMA and other peaks on the chromatogram but is extracted and oxidized much like VMA, thus minimizing the effects of minor variations of recovery or detector voltage. About 70% of VMA is carried through the assay of urine samples whereas about 80% of EMA is carried through. This ratio is constant and can be used to calculate recovery of VMA in any urine sample to which EMA has been added. Representative chromatograms showing endogenous VMA and urine with added VMA are shown in Fig. 3.

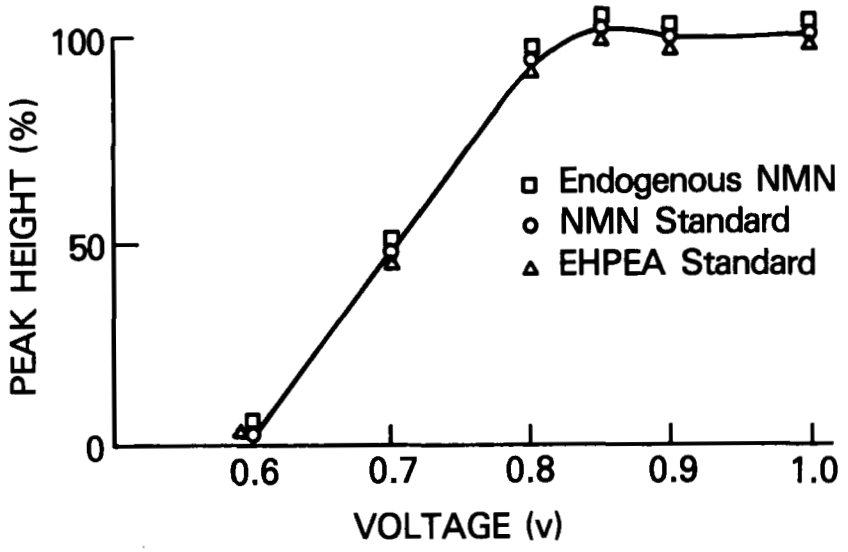


FIGURE 2. Relative Detector Response as a Function of Oxidizing Potential vs. Ag/AgCl Electrode for Endogenous NMN, NMN Standard and EHPEA Standard



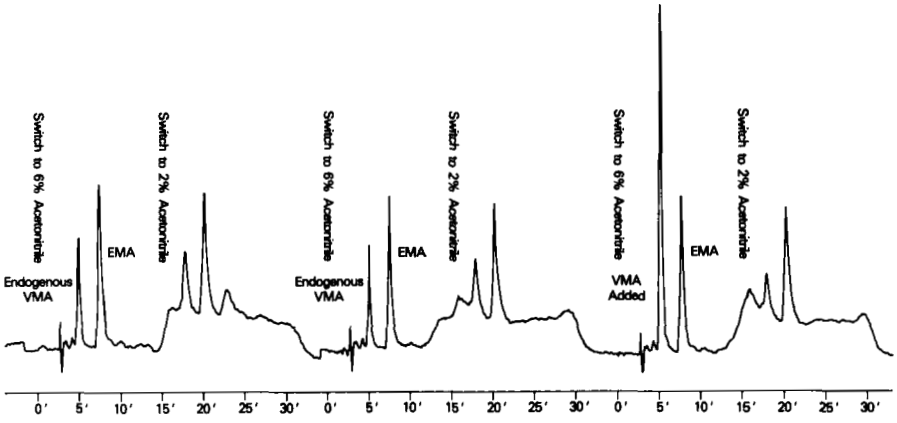


FIGURE 3. Typical Duplicate Chromatogram of VMA in Human Urine with Only EMA Added, and Repeated with Addition of VMA Standard

NMN: The method used here was essentially that reported by Jackman (13), except that smaller volumes of urine were used, and borate buffer at pH 9.0 was found to be more optimal for extraction of NMN than pH 10.0 used previously. Like the homologous internal standards for VMA and MHPG, EHPEA is a suitable internal standard for NMN because of good separation on chromatography, and similiar behavior, both in sample purification and in electrochemical detection. Consistency of the ratio of added EHPEA and NMN carried through the assay allows for estimation of recovery of NMN in each sample, with about 40% of added NMN and 45% of added EHPEA being carried through the procedure. A typical chromatogram of endogenous NMN, and an additional sample with added NMN is shown in Fig. 4.



## Calculation of Urinary VMA and NMN

Urinary VMA and NMN were determined from peak heights of endogenous metabolites and their respective internal standards, in conjunction with peak heights of known amounts of standards. Correction factors for ratio of recoveries of the metabolites and internal standards were determined in each batch of urine samples from several additional samples to which additional metabolite had been added.

## Precision

For 34 duplicate samples, values obtained for VMA agreed within an average of 2.4% (range: 0-6.8%). Values obtained on three samples run on separate days differed by an average of 4.9% (range: 3.1-6.5%). For NMN, 31 samples run in duplicate had an average difference of 4.5% (range: 0.4-9.9%). Between-run precision was determined on three samples run on separate days. These values agreed within an average of 6.8% (range: 3.0-8.8%).

## Accuracy

Each of these methods was compared with a GCMS assay to determine accuracy of the procedures. For VMA, a correlation

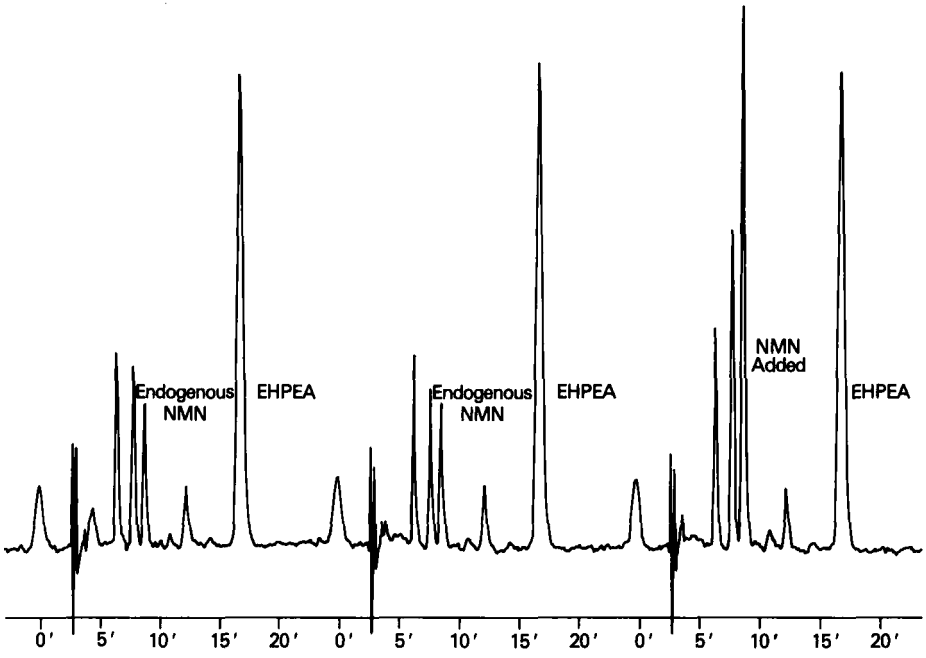


FIGURE 4. Typical Duplicate Chromatogram of NMN in Human Urine with Only EHPEA Added, and Repeated with Addition of NMN Standard.



coefficient of 0.995 was obtained with a regression equation of $y = 1.01x - 0.0780$, on ten paired samples having a concentration range of 0.89-3.81 micrograms/ml. Comparison of values for NMN on nine paired samples with a concentration range of 38-128 ng/ml gave a correlation coefficient of 0.972 and a regression equation of $y = 0.933x + 4.32$.

## Conclusion

The ethoxy-homologs of VMA and NMN serve as useful internal standards for measurement of urinary levels of these metabolites by HPLC, without interfering peaks, and with accuracy comparable to GCMS when used in the assays described here.

## ACKNOWLEDGMENTS

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