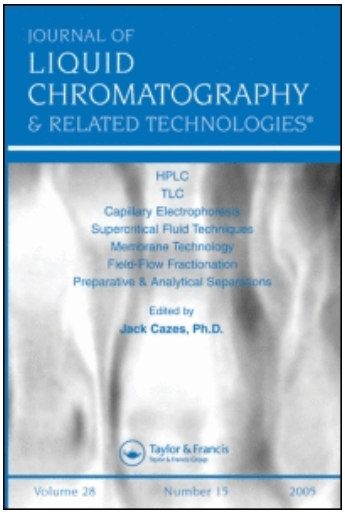

Journal of Liquid Chromatography & Related Technologies
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713597273

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To cite this Article Kumar, Adarsh M. , Kumar, Mahendra , Fernandez, Benny J. , Goodkin, Karl , Schneiderman, Neil and Eisdorfer, Carl(1995) 'An Isocratic HPLC-ECD Assay of Urinary Normetanephrine and Metanephrine', Journal of Liquid Chromatography & Related Technologies, 18: 11, 2257 — 2269
To link to this Article: DOI: 10.1080/10826079508010269
URL: http://dx.doi.org/10.1080/10826079508010269

JOURNAL OF LIQUID CHROMATOGRAPHY, 18(11), 2257-2269 (1995)

AN ISOCRATIC HPLC-ECD ASSAY OF URINARY NORMETANEPHRINE AND METANEPHRINE

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ABSTRACT

This report describes a procedure to quantify simultaneously urinary normetanephrine (NMTN) and metanephrine (MTN) using an isocratic HPLC-ECD methodology. An aliquot of urine (5ml) was adjusted to pH of 1.0, spiked with a known concentration of internal standard, (3-methoxy-4-hydroxy benzylamine, MHBA) and the two metabolites were hydrolyzed in a boiling water bath. The metabolites were adsorbed on a Biorex-70 column and eluted with ammonium hydroxide. Final extraction was carried out in a mixture of ethyl acetate and acetone(2:1, v/v). After drying the extract under nitrogen, it was dissolved in mobile phase, filtered through 0.2μ filters, and injected into a 4 μ Nova-Pak, C_{18} column of the HPLC system. Mobil phase for elution contained citric acid, sodium acetate, EDTA-Na_2, sod. octyl sulfate, dibutylamine, methanol 2% and isopropanol 2%. Peaks were detected by the electrochemical detector at a potential of + 0.55V and characterized using the retention times obtained from HPLC profiles of the standards. Calibration of HPLC was performed by spiking 5 ml of metabolite free urine (MFU) with known amount of standards of NMTN, MTN and MHBA and injecting the extract to obtain chromatographic profile. Concentrations of the metabolites were calculated on a pre-programmed data module using ratio of the areas of the analytes to that of the internal standard. This assay showed a linear relationship between 5-80 ng/ml for NMTN and 5-90 ng/ml MTN. Sensitivity of the method was below 5.0 ng/ml. The total elution time was 15 min. Six to eight urine samples could be extracted and assayed in one day.

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INTRODUCTION

It is now well recognized that in order to investigate the total body catecholamine excretion, urinary catecholamines and their metabolites should be assayed. Metanephrine, normetanephrine and 3-methoxytyramine are the basic metabolites of catecholamines: epinephrine, norepinephrine and dopamine, respectively. These metabolites along with other metabolites of catecholamines, viz., vanillylmandelic acid (VMA) and 3- methoxy- 4-hydroxyphenylglycol (MHPG) account for over 90% of the total catecholamine metabolites (1,2) and are excreted in urine. Determination of urinary metanephrines gives the best measure of both the central and peripheral metabolism of catecholamines and has been helpful in the diagnosis of pheochromocytoma, neuroblastoma and other diseases of neural crest origin (3,4,5). More recently, determination of urinary metanephrine and normetanephrine has also been used in the diagnosis of hypertension (6,7), myocardial infarction (8), and muscular dystrophy (9) since these disease states bring about various changes in urinary excretion of catecholamines and their metabolites. For example, studies show that the mean excretion rate of normetanephrine by hypertensive individuals is greater (upto 600 μg/24 hrs) than that of the normal persons (upto 300μg/24 hrs) (10). In addition, urinary metanephrines and normetaneprine determinations may be valuable in differentiating patients with affective disorders (11,12). To detect relatively small changes in the concentration of these metabolites it is essential to quantify reliably the normal range of urinary metanephrine and normetanephrine concentrations. The spectrophotometric procedures used earlier to determine urinary metanephrines were not sufficiently specific or sensitive to quantify samples having low levels of metanephrine concentrations. Methods involving gas chromatography-mass spectrometry and mass fragmentography (13,14,10) are highly specific and sensitive but involve very expensive equipment and are time consuming. Assay methods involving high performance liquid chromatography equipped with the electrochemical detector (HPLC-ECD) have become available and provide speed and accuracy in the determination of very small

quantities of these compounds present in biological fluids. In our earlier publications, we described HPLC-ECD techniques to assay urinary norepinephrine, epinephrine, dopamine, MHPG and VMA (15,16). We describe here a highly sensitive and specific isocratic HPLC-ECD assay procedure for the determination of nanogram quantities of metanephrines in urine.

MATERIAL AND METHODS

Chemicals and reagents

Normetanephrine hydrochloride and metanephrine hydrochloride were purchased from Sigma Chemical Co. (St. Louis, MO). 3-methoxy-4-hydroxybenzylamine and sodium octylsulfate were obtained from Aldrich Chemical Co. (Milwaukee, WI). Biorex-70 ion exchange columns were purchased from the Biorad Laboratories (Richmond, CA). Sodium acetate, citric acid, dibutylamine, ethylenediamine tetracetate (NA_2EDTA) were obtained from Eastman Kodak Co, (Rochester, NY). All other chemicals were of HPLC grade and were obtained locally.

HPLC Equipment

The liquid chromatography (HPLC) system (Water's Associate, Milford MA) consisted of an injector (Model U6K), solvent delivery system (Model 590) and an electrochemical detector (Model 460), equipped with a glassy carbon working and auxiliary electrodes and silver-silver chloride reference electrode. Water's data module 740 was used to integrate the peaks and calculate concentrations of metabolites in samples on the basis of prior calibration with known amount of standards. The chromatography column 4μ Novapak, C_{18} reversed phase, 3.5 cm x 150 mm (Water's Associate) was used.

Standard Solutions

Stock solutions (1 mg/ml) of normetanephrine (NMTN), metanephrine (MTN) and 3-methoxy 4-hydroxybenzylamine (MHBA) were prepared in HPLC grade water. Stock solution of each standard was divided into 1-2 ml aliquots and kept frozen at -80°C. Frozen standards

were thawed, and diluted with water just before use to prepare aqueous working standard mixture containing in 20 µl 0.4 ng NMTN, 0.8 ng MTN, and 1.2 ng internal standard, MHBA. Internal standard MHBA was added to urine samples before hydrolysis.

Urine Collection

Urine samples either 24 hours or spot collections were obtained and acidified to maintain pH< 2.0 with 1N HCL.

Metanephrine-free urine

In order to obtain a metanephrine-free urine (MFU), a pooled sample of urine was adjusted to pH 10.6 (metanephrines are unstable at alkaline pH) and exposed to air and light for at least 5 days for degradation of metabolites. The pH was then adjusted to 6.5 and the sample was centrifuged at 15,000 rpm for 10 minutes. From the supernate an aliquot (5 ml) was taken and used for extraction and carried through all the steps given below for hydrolysis and extraction of metanephrines from urine samples. The final extract when injected showed a HPLC profile without any detectable peak of either NMTN or MTN. The pool supernate obtained after centrifugation of MFU was then divided into 5ml aliquots and stored at -80°C and used whenever required for calibration. Another 5 ml aliquot of MFU was then spiked with known concentrations of standards for calibration of the instrument and treated similar to the samples.

Mobile Phase

Elution of metanephrines from the HPLC column was achieved using a mobil phase of the following composition: Sodium acetate 0.05M, citric acid 0.05M, sodium octyl sulfate 0.5mM, Na_2EDTA 0.075M, dibutylamine 0.5mM, methanol 2% and isopropanol 2%. The solution was adjusted to pH 3.5, filtered through 0.45 µm filter and degassed by sonication for 30 seconds at room temperature before use.

Extraction Procedure

Metanephrines are excreted in urine in conjugated form either as glucuronides or sulfates. They must be acid hydrolysed before they can be adsorbed on the resin column used to remove interfering substances present in urine. To hydrolyse the conjugated compounds in urine a 5ml aliquot of urine was placed in a screw cap tube, internal standard solution 50µl containing 600 ng of MHBA was added and pH adjusted to 1.0 with concentrated hydrochloric acid. The tubes were placed in a boiling water bath for 30 minutes and then were cooled to room temperature. After centrifugation at 4000 rpm for 5 minutes the pH was adjusted to 6.5 and hydrolysed urine samples were diluted with 15 ml phosphate buffer, pH 6.5, run through cation exchange, Biorex-70, columns and allowed to drain completely. Columns were washed twice with 5ml distilled water. Catecholamines were eluted with 5 ml of 4% boric acid. After the columns ceased to drip the eluates they were washed again twice with 5ml distilled water. The columns were then placed over a screw-cap tube containing 2.5g NaCl. Metanephrines were eluted with 5ml of 4N ammoniuim hydroxide into the screw cap tube. The eluate was extracted twice with 4 ml of a mixture of ethylacetate: acetone (2:1 v/v). Extraction was performed by mixing on a shaker for 10 minutes. After centrifugation, the organic layer from both of the extractions was pooled and 4ml aliquot was evaporated to dryness at 40^{0}C under a stream of nitrogen gas as described by Shoup and Kissinger (17). The residue was reconstituted with mobile phase (200µl), vortex mixed and allowed to stand for 30 minutes. The extract was filtered through 0.2µ filter (Whatman filters) attached to a syringe and diluted 1:10 with mobile phase. Injections of 20µl of the diluted extract were applied to the HPLC system.

Calibration of HPLC with MFU spiked with standards

Calibration of HPLC was performed by spiking a 5 ml aliquot of MFU. Frozen MFU was thawed and 50 µl of a mixture containing 200ng NMTN, 400ng MTN, and 600ng MHBA

was added. Hydrolysis and extraction procedures were followed as described above for the urine samples. The extracted residue was dissolved in 200 µl mobil phase, filtered through the syringe filter, diluted 1:10 and 20µl aliquot was injected to obtain discrete peaks for calibration. The data module was programmed for calibration using the known concentrations of NMTN, MTN and MHBA and the area of their peaks. Another 20µl injection of the same extract was used to ascertain stability and reproducibility of retention times. Based on calibration, calculation of metabolite concentrations (ng/ml) in urine samples were performed by ratio method, where area of peak of internal standard (MHBA) to that of sample is used to calculate the concentration of analytes in the sample.

Extracts from urine samples were then injected and based on the previous calibration, calculations were made by the data module using the ratio of the integrated areas of peaks of individual analytes to that of internal standard, MHBA of known concentration.

Chromatographic analysis were validated and linearity curves were prepared with a series of aqueous standards of concentrations ranging from 5ng-100ng/ml of both NMTN and MTN.

RESULTS AND DISCUSSION

Typical elution profiles of NMTN, MTN and MHBA are shown in the chromatograms presented in Fig. 1 (A,B, C and D). The retention times of NMTN, MTN and MHBA were found to be 8.319, 10.655 and 16.557 minutes respectively (Fig. 1A). An aliquot of MFU was taken through the whole assay procedure. The extract so obtained was diluted, and 20µl of this extract was injected into the HPLC.

The method described in this paper includes important modifications for improving the sensiyivity as well as specificity over the procedures described earlier (17,18,19,20). In our procedure we used 4µ Novapak C_{18} column and a diffetent mobile phase for the separation of NMTN, MTN and internal standard MHBA than that used by Shoup and Kissinger (17) and

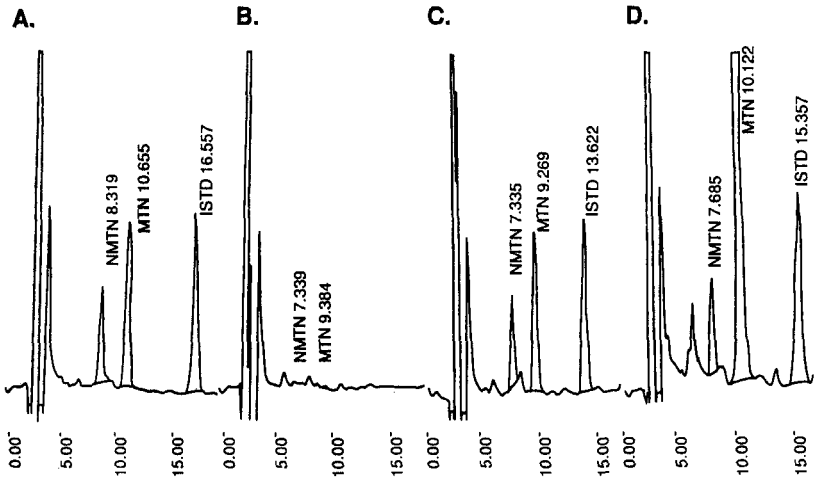

Fig 1: HPLC chromatograms showing peaks with their respective retention times for NMTN, MTN and internal standard. MHBA aqueous standards of known concentrations (A) NMTN and MTN free urine (MFU) extract (B), MFU spiked with known concentrations of standards (C), profile of extract from a urine sample (D).

by Orsulak et al (18). The quality of chromatogrphic profiles in Fig. 1A-1D show well separated peaks and good resolution of analytes of interest. The peak of MTN is separated from NMTN by two minutes and MHBA is separated from MTN by approximately four minutes. Although the total time of resolution and order of appearance of peaks of NMTN, MTN and MHBA are similar to the one obtained by Radial-pak C_8 cartridge used by Orsulak (18) our chromatograms show a clear separation of analytes due to increased differences in retention times. Furthermore, there were no other discrete peaks between NMTN and MTN and no other peaks such as tyramine appeared after the internal standard.

The chromatographic profile of MFU extract shows (Fig. 1B) essentially the baseline with some noise and no identifiable peaks of either NMTN or MTN or any other interfering substances. Fig 1C shows the profile of MFU spiked with known concentration of NMTN, MTN and MHBA. A chromatographic profile of the extract obtained from an aliquot of a

spot collection of a urine sample and taken through the entire extraction procedure is shown in Fig. 1D. The characterstic peaks for NMTN, MTN and MHBA obtained from this extract had retention times in the same range as obtained with those for aqueous standards given in Fig. 1A.

Standard curves for NMTN and MTN were generated and linearity was established by spiking a wide range of concentrations of NMTN and MTN (5-100 ng/ml) and a constant concentration (60 ng/ml) of MHBA to a 5 ml aliquot of MFU. After the assay procedure involving isolation of metanephrines by ion exchange columns and extraction with a mixture of ethylacetate-acetone the diluted extract was injected and calibration curves were obtained by plotting the ratios of the areas of NMTN and MTN to that of MHBA against the corresponding concentrations of each analyte. As shown in plot (fig. 2) the detector sensitivity for NMTN and MTN was identical at lower concentrations (5-20 ng/ml) whereas with increasing concentration (20-80 ng/ml) the detector sensitivity for MTN was higher than that for NMTN. However, the curves for NMTN and MTN plateaued at a concentration of 80 and 90 ng/ml respectively.

The data from the linearity of standards suggest that in cases where levels of these metabolites are expected to be high in urine, such as in some disease states, the starting volume of urine should be reduced, or, alternatively, the final extract should be diluted more before injection into HPLC to maintain the detector sensitivity within the linear range of detectable concentration (5-80 ng/ml).

HPLC analysis of all diluted extracts was carried out at a potential set at +.55V and sensitivity at 0.2 nAFs with a flow rate of mobile phase maintained at 0.5 ml/minute. This potential was found to be optimum after investigating the responses of both NMTN and MTN at different potentials (+ 0.45 to ± .65, data not given). The pH of mobile phase ranging between 3.35 to 5.0 was tested to evaluate the optimal pH for eluting the analytes. The most

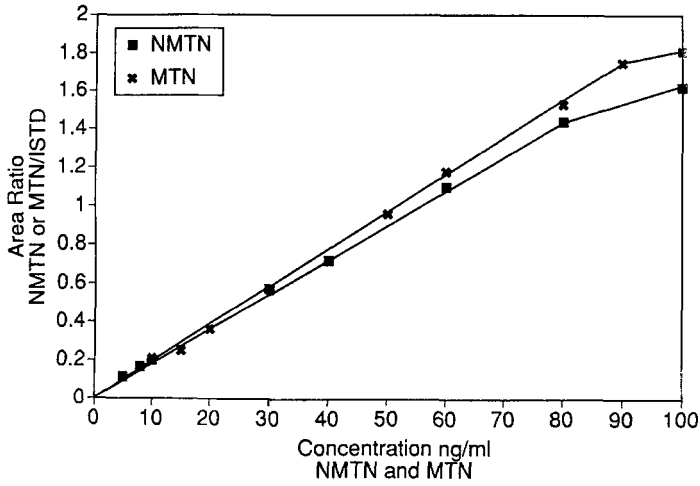

Fig 2: Standard curve of NMTN and MTN showing a linear relationship between various concentrations of NMTN (5- 80 ng/ml) and MTN (5- 90 ng/ml) and the ratio of the areas of peaks of NMTN and MTN to that of internal standard (MHBA).

effective pH was 3.5, which was maintained throughout the analysis. Isopropanol 2% and methanol 2% in mobile phase were found to be a good combination to obtain sharp and well separated peaks of analytes with stable retention times. The use of a detergent, sodium octylsulfate 0.5 mM, provided further stability and high efficiency in separating the peaks, and maintained the order of retention time of the compounds being eluted such as the peak of MHBA always appearing after those of NMTN and MTN. Before injections of aqueous standards or extracts of samples the mobile phase was allowed to flow for at least 4-6 hours to equilibrate the column and to stabilize the detector response. Occasionally a new lot of mobile phase was pumped overnight and recirculated but was discarded when samples were analysed.

Addition of 2.5g NaCl to the aqueous solution before extraction is an important step for increasing the recovery since it aids in the "salting out" of the aqueous phase, and in the

solute transfer to organic phase. Using ethylacetate in combination with acetone (2:1, v/v) enhances extraction due to polarization of ethylacetate by acetone. Recoveries were also optimized by the solvent volume used for extraction: thust two aliquots of 4 ml of solvent mixture, compared to one 8ml aliquot was found to be better for good recovery of various concentration of compounds added for standard curve, apparently due to a better distribution of analytes in the solvent mixture. Addition of internal standard to samples before extraction normalized the recoveries and improved precision and provided a convenient and consistent method for calculating the results in the data module. Any variability due to transient changes in column performance, in the detector sensitivity because of changes in electrical impulses, or temperature variation is also reduced, since both the internal standard and the analytes will undergo parallel changes at the same time.

Within run (intraassay) chromatographic determinations (N=7) for normetanephrine 20 ng/ml and metanephrine (40 ng/ml) gave a coefficient of variation of 7.23 and 4.72% respectively. When aliquots (N=8) of the same sample were extracted separately (interassay), a coefficient of variation for NMTN (20 ng/ml) and MTN (40 ng/ml) was 8.8 and 6.7%, respectively. Analysis of spot collected urine from normal subjects with no dietary restrictions gave values of NMTN 11.9±.64 and MTN 253.9±2.12 ng/ml urine.

One of the salient features of this method is the improvement in sensitivity for detecting the analytes of interest. The sensitivity of the present method is higher than described in earlier methods by Shoup and Kissinger (17) and by Orsulak et al. (18), about several hundred fold higher than that reported by Dutrieu et al (19) and Flood et al (20). This increase in sensitivity was achieved by using a different HPLC column (4μ Novapak C_{18}), and a new mobile phase for eluting the analytes. Another salient feature of this method is that the concentration of metanephrines were measured by the internal calibration technique using MFU devised in our laboratory. This method of calibration and standardization, where MFU

is spiked with known concentration of standards offers a simplified way of calculating the results as compared to the standard addition technique used in earlier studies (18) where urine containing metanephrines is further spiked with standard and the values are calculted by difference. An aliquot of MFU was always tested before spiking with standards. A sample of MFU was discarded if chromatogram showed any peaks, since these peaks can interfere in the calculation of the final results. The procedure described provides simultaneous analysis of normetanephrine and metanephrine in a single chromatogram run in 15 minutes. As many as six to eight samples can be extracted concurrently and be analysed chromatographically in one day. Samples containing as low as 10 ng/ml and as high as 80 ng/ml of both NMTN and MTN can be measured by this method. This increased sensitivity may be helpful in distinguishing normal subjects from those with more subtle derangements in catecholamine metabolism.

ACKNOWLEDGEMENT

This investigation was supported, in part by grants #HL536588, MHPO149548 and MH48628.

REFERENCES

1. Axelrod, J., Inscoe JK, Senoh, S., & Witkop B: O-mehylation, the principal pathway for the metabolism of epinephrine and norepinephrine in the rat. Biochem.Biophys. Acta. 27:210, 1958.

2. LaBrosse, E.H. & Mann, J.D.: Presence of metanephrine and normetaphrine in normal human urine. Nature, Lond. 185:40, 1960

3. Pisano, J.J. A simple analysis for normetanephrine and metanephrine in urine. Clin. Chim. Acta 5:406-414, 1960.

4. Greer, M., Sprinkle T.J., Williams, C.M.: Determination of urinary 3-methoxy-tyramine, normetanephrine, and metanephrine in pheochromocytoma and neuroblastoma by gas chromatography. Clin. Chim. Acta, 21:247, 1968.

5. Moyer, T.P., Jiang, H.S., Tyce, G.M., and Sheps S.G. Analysis for urinary catecholamines by liquid chromatography with amperometric detection: Methodology and clinical interpretations of results. Clin Chem. 25:256-263, 1979.

6. Ruthven, C.R.J., Sandler, M: The estimation of 4-hydroxy-3 methoxyphenylglycol and total metadrenalines in human urine. Clin Chimica. Acta., 12:318-324, 1965.

7. Naftchi, N.E., Wooten, G.F., Lowman, E.W. and Axelrod J: Relationship between serum β-hydroxylase activity catecholamine metabolism and hemodynamic changes during paroxysmal hypertension in quadriplegia. Cir. Res. 35:850, 1974.

8. Hingerty D., and O'Boyle, A: Clinical Chemistry of the Adrenal Medulla, Charles C. Thomas, Springfield, IL, 1972.

9. Kabara, J.J., Riggin, R.N., and Kissinger P.T.: Abnormal levels of urinary catecholamines in dysphoric mice and hamsters. Proc. Soc. Exp. Biol, Med. 151:168, 1976.

10. Canfell, C., Binder, S.R., and Khajam-Bashi, H: Quantitation of urinary normetanephrine and metanephrine by reversed phase extraction and mass-fragmentographic analysis. Clin Chem 28:25-28, 1982.

11. Shildkraut, J.J., Orsulak, P.J., Schatzberg, A.F., Gudeman, J.E., Cole, J.E., Rhode, W.A., and LaBrie, R.A.: Towards a biochemical classification of depressive disorders 1: Differences in urinary MHPG and other catecholamine metabolites, in clinically defined subtypes of depression. Arch Gen Psychiatry, 35:1427-1433, 1978.

12. Shildkraut, J.J., Orsulak, P.J., Labrie, R.A., Schatzberg, A.F., Guderman, J.E., Cole, J.E., Rohode, W.A.: Towards a biochemical classification of depressive disorders 11: Application of multiple discriminant function analysis to data on urinary catecholamines and metabolites. Arch Gen Psychiatry 35:1436-1439, 1978.

13. Robertson, D., Heath E.C., Falkner, F.C. et al: A selective and sensitive assay of urinary metanephrine and normetanephrine using gas chromatography with selected ion monitoring. Biomed. Mass. Spectrom 12:704-708, 1978

14. Muskiet F.A.J., Thamasson CG, Gerding, A.M., Fremouw-Otterangers, D.C., Nagel, T.G., and Wolthers, B.G.: Determination of catecholamines and their 3-0-methylated metabolites in urine by mass fragmentography with use of deuterated internal standards. Clin. Chem. 25:453-460, 1979.

15. Kumar, A.M., Kumar M., Fernandez, J.B., Mellman, T.A., and Eisdorfer C: A simplified HPLC-ECD technique for measurement of urinary free catcholamines. J Liq Chromatography. 14(19):3547-3557, 1991.

16. Kumar, A.M., Kumar, M., Fernandez, J.B., Schneiderman, N., and Eisdorfer, C.: an improved assay of urinary catecholamines metabolites, 3-methoxy-4-hydroxyphenylglycol and vanillylmandelic acid, using high performance liquid chromatography with electrochemical detection. J. Liq. Chromatography. 16(6):1329-1340, 1993.

17. Shoup, R.E., Kissinger, P.T.: Determination of urinary normetanephrine, metanephrine, and 3-methoxytramine by liquid chromatography with amperometric detection. Clin Chem 23:1268-1274, 1977.

18. Orsulak, P.J., Kizuka, P., Grab, E., and Shildkraut, J.: Determination of urinary normetanephrine and metanephrine by radial compression liquid chromatography and electrochemical detection. Clinical Chemistry 29: 305-309, 1983.

19. Dutrieu, J., and delmtte, Y. E.: LCEC-Determination of urinary normetanephrine, metanephrine and 3-methoxytyramine. Fresenius Z. Anal Chem. 316:505-508, 1983.

20. Flood, J.G., and McComb R.B.: Urinary metanephrines as measured by liquid chromatography with an on line post-column reaction detector. Clin Chem 27:1268-1271, 1981.

21. Bertani-Dziedzic, L.M., Krstulovic, A.M., Dziedzic, S.W., Gitlow, S.E., and Cerqueria. Analysis of urinary metanephrines by reversed-phase high-performance liquid chromatography and electrochemical detection. Clin Chim Acta 110:1-8, 1980.

Received: August 8, 1994
Accepted: January 24, 1995