Evaluation of a urinary metanephrines reagent kit: an automated approach

Thevarajah M T, Nadarajah S, Chew Y Y, Chan P C

ABSTRACT

Introduction: We report the results of a laboratory evaluation of the BioRad Urinary Metanephrines Reagent Kit. The test was designed for the quantitative measurement of normetanephrine and metanephrine in urine by high performance liquid chromatography. The kit was evaluated in view of improving assay reliability and specificity as compared to the manual method based on cation exchange chromatography and spectrophotometry.

Methods: Performance was evaluated for precision, linearity, accuracy, sensitivity and detection limit based on National Committee on Clinical Laboratory Standards (NCCLS) protocols. Analytical precision was evaluated using commercial controls and patient sample. Accuracy was evaluated by assessing recovery. Linearity was determined using aqueous standards.

Results: The within-run coefficient of variation (CV) for metanephrine and normetanephrine were 1.9 percent and 2.4 percent (low control); 4.2 percent and 3.5 percent (high control); 3.8 percent and 3.3 percent (patient sample), respectively. The between-day precisions were 3.8 percent and 4.3 percent (low control); and 5.5 percent and 3.7 percent (high control) for metanephrine and normetanephrine, respectively. The linearity curve showed metanephrine and normetanephrine to be linear with concentrations, to at least 1,600 microgramme per litre and 2,000 microgramme per litre, respectively. Analytical recovery averaged 102 percent for metanephrine and 95 percent for normetanephrine. Levels as low as 23 microgramme per litre normetanephrine and 10 microgramme per litre metanephrine were measured with this method. The detection limit was 3.3 microgramme per litre for metanephrine.

Conclusion: The performance characteristics of automated sample preparation and auto-injection facilitate handling of larger number of samples as well as improve assay reliability.

Keywords: automated method reagent kit, high performance liquid chromatography, urinary metanephrine, urinary normetanephrine

Singapore Med J 2008; 49(6): 454-457

INTRODUCTION

Determination of urinary metanephrine (MN) and normetanephrine (NMN), collectively referred to as urinary total metanephrines, remain reliable parameters for the diagnosis of pheochromocytoma.[1-4] Traditional spectrophotometric methods[5,6] have now been superseded by high pressure liquid chromatography (HPLC) techniques[7-10] that enable separation of urinary metanephrines into their fractionated components (MN and NMN). Measurements of fractionated metabolites allow better detection of tumours that produce predominantly, or only, one of the metabolites. However, sample preparation and the extraction of metanephrines from urine[8-11] for HPLC procedures are relatively slow and tedious. Automation of these steps will simplify and increase the robustness of the HPLC procedures by reducing the rate of sample throughput while enhancing assay precision. In view of this, we evaluated the Bio-Rad Urinary Metanephrines Reagent Kit by HPLC with automated sample preparation on the Gilson ASPEC™ Solid Phase Extraction System (Gilson Inc, Middleton, WI, USA).

METHODS

The detailed information on test procedures, operation of sample processor (Gilson ASPEC™), HPLC system specification and analysis is fully covered in the Bio-Rad instruction manual.[12] The following is an overview of the test procedures involved. All reagents (hydrolysis, basic, acidic, reconstitution, dilution, transfer buffer and indicator reagent), mobile phase, internal standard, analytical cartridge, guard cartridge and calibrator used were supplied in the Bio-Rad Urinary Metanephrines by HPLC – Automated Method Reagent Kit. For quality control and precision run, quantitative urine controls for normal and abnormal statuses (Lypochek, Bio Rad, Anaheim, CA, USA) were used, each reconstituted with 10 ml of 0.05 N HCl, aliquoted and stored at -20°C. 24-hour urine samples used in the precision and recovery study were collected in 10 ml 6 N HCl, aliquoted and stored at

Department of Pathology, Faculty of Medicine, University of Malaya, Lembah Pantai, Kuala Lumpur 50603, Malaysia

Malathi T, MBBS, MPath
Senior Lecturer and Chemical Pathologist

Clinical Diagnostic Laboratory, University of Malaya Medical Centre

Nadarajah S, BSc
Medical Laboratory Technologist

Chew YY, BSc
Senior Biochemist

Chan PC, BSc
Medical Laboratory Technologist

Correspondence to:
Dr Malathi T Thevarajah
Tel: (60) 3 7949 2745
Fax: (60) 3 7949 2818
Email: tmalathi@um.edu.my

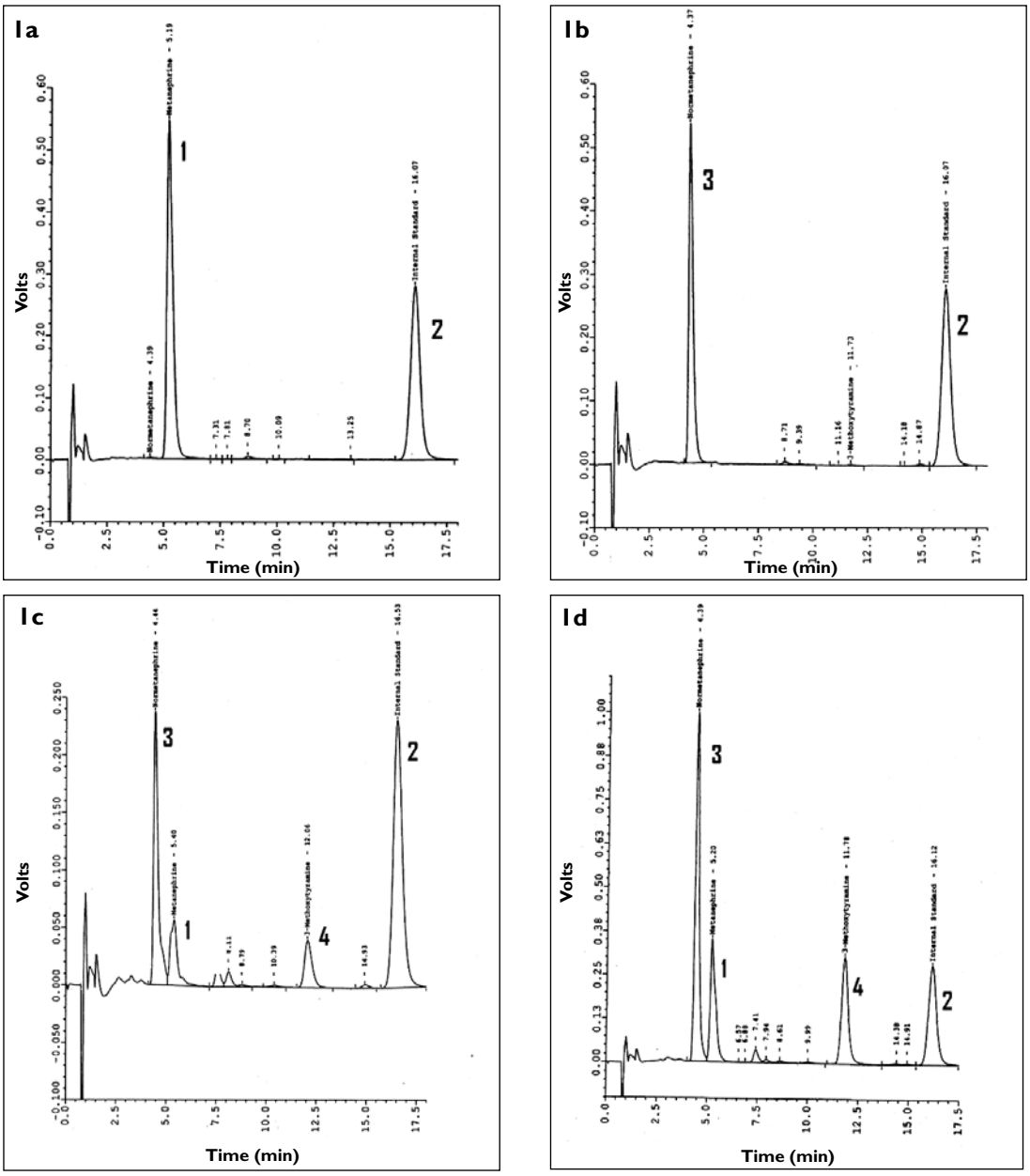

Fig. 1 Representative chromatograms of injections show (a) metanephrine standard; (b) normetanephrine standard; (c) urine sample; and (d) Bio-Rad abnormal urine control. Peaks indicate: 1: metanephrine; 2: internal standard; 3: normetanephrine; and 4: methoxytyramine. Unlabelled peaks are extraneous.

-20°C. A pH of between 1.5 and 6.5 was required for the correct determination of metanephrines.

For hydrolysis, 500 µL of urine, calibrator and controls were pipetted into ten 75-mm disposable glass tubes. 500 µL distilled water, 50 µL internal standard and 75 µL hydrolysis reagent were added to each tube and mixed well. All tubes were covered with aluminium foil. The tubes were placed in a dry block heater for complete hydrolysis of metanephrines. All tubes were left to cool to room temperature. The aluminum foil was removed, and the contents were mixed well before the samples were loaded into the sample processor for solid phase extraction.

In the solid phase extraction, the automated steps involved the addition of internal standard and dilution of hydrolysed sample with dilution reagent, after which the mixture was applied onto a cation exchange column. Excess sample and interfering substances were washed off the column with deionised water. The column was eluted with transfer buffer and this elute was applied directly onto an anionic exchange column. After washing the column with distilled water, metanephrines were eluted with elution reagent. The elute was acidified with acidic reagent. A diluted aliquot of this elute was injected into an isocratic HPLC system. After analysis was performed, the indicator reagent was added to each hydrolysate to check the pH value. Hydrolysates showing yellow-green, green or blue-green colours indicated a correct pH value of the used urine. Hydrolysates showing a yellow colour had a pH < 4, indicating that the urine sample was too acidic. Hydrolystes showing a violet colour had a pH > 7.5, indicating that the urine sample was too alkaline. Those samples had to be re-analysed with a correct pH.

For chromatography, we used a Model 307 Pump

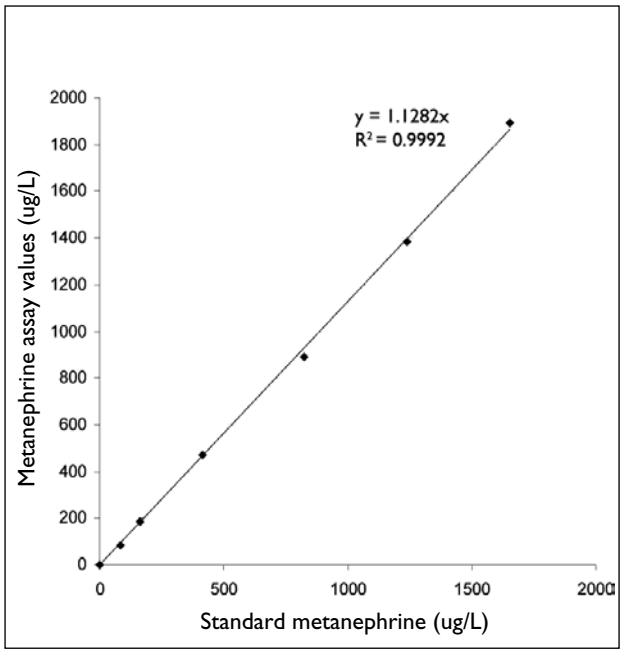

Fig. 2 Linear curve of metanephrine.

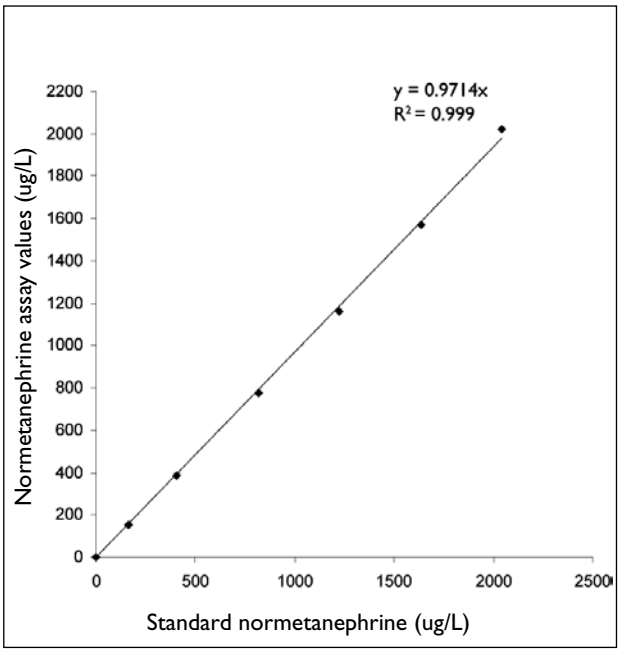

Fig. 3 Linear curve of normetanephrine.

(Gilson Inc, Middleton, WI, USA), Reversed Phase Analytical Cartridge with Guard Cartridge (supplied as part of the Bio-Rad Urinary Metanephrines Reagent Kit), sample injector (Gilson ASPEC XL with Model 402 Dual Syringe Pump [Gilson Inc, Middleton WI, USA]), injection loop 20 µL, a Model 1640 Electrochemical Detector (Bio-Rad), integrator (Bio-Rad Clinical Data Management interface and software) and column heater (Pickering Laboratories, Mountain View, CA, USA). Instrumental conditions were: column flow rate 0.5 ml/min; column heater temperature 58°C; electrochemical detector, potential +0.65 V, sensitivity 10 nA/V; integrator, duration of data acquisition approximately 17 minutes.

Recording of peaks and automatic calculation was facilitated by the integration software (Bio-Rad Clinical Data Management Software). Peak height ratio (phr) of MN and internal standard (IS) of the calibrator (CAL) and unknown sample were used for calculation:

$$\text{MN concentration (MNC) of unknown sample} = \frac{\text{MNC (CAL)}}{\text{MN/IS phr (CAL)}} \times \text{MN/IS phr of unknown sample}$$

The example was valid for MN. The same formula was applied for NMN.

RESULTS

The typical chromatograms of MN and NMN standards, abnormal urine control and actual urine sample, are shown in Fig.1. This method allows additional measurements of methoxytyramine, the O-methylated metabolite of dopamine, which is seen as the extra peak (peak 4) in the urine chromatograms. We did not evaluate the performance characteristics of methoxytyramine since our laboratory, did not report on it. The chromatograms of the control and urine samples are clean with few extraneous peaks. This indicates that the extraction procedure is satisfactory. However, interferences are to be expected from substances that are retained, eluted, having the same retention time and electrochemical activity as MN and NMN. Salsolinol is known to cause interfering peaks at high concentration.[12]

To check precision, Lypochek Quantitative Urine Control Normal and Abnormal (Lypocheck, Bio-Rad, Anaheim, CA, USA) was used. Within-run precision was evaluated by analysing 20 replicates each for the low-control, high-control and unknown urine samples. Between-day precision was evaluated by analysing low control and high control in duplicates over ten days. The results showed acceptable precision and accuracy, and compared well with mean values reported in Bio-Rad's product insert (Table I). To determine linearity, aqueous standards of six concentration levels of NMN and MN were prepared. The linearity curve (Figs. 2 & 3) showed MN to be linear with a concentration to at least 1,600 µg/L and NMN to be linear with a concentration to at least 2,000 µg/L.

To determine analytical recovery, samples from a urine pool with known concentrations of NMN and MN were spiked with three concentration levels. Four replicates for each level were analysed. The results showed acceptable recovery for both MN and NMN for the concentration levels tested (Table II). To evaluate sensitivity, normal urine control was diluted 1:9 with saline to obtain levels 10.2 µg/L for MN and 30.5 µg/L for NMN. Ten replicates of each diluted sample were analysed in a single run, Levels as low as 10 µg/L MN and 23 µg/L NMN were measured at sensitivity 10 nA/V of the electrochemical

Table I. Precision of the method.

	Metanephrine		Normetanephrine	
	Mean (μg/L)	CV (%)	Mean (μg/L)	CV (%)
Lypochek normal	102 (77–127)[a]		305 (229–381)[a]	
Within run	106	1.9	297	2.4
Between day	106	3.8	325	4.3
Lypochek abnormal	545 (410–680)[a]		1,350 (1,080–1,620)[a]	
Within run	599	4.2	1,398	3.5
Between day	603	5.5	1,377	3.7
Patient sample				
Within run	158	3.8	420	3.3

[a] Mean values (and ranges) obtained by HPLC as reported in Bio-Rad's product insert.

Table II. Recovery of the method.

	Metanephrine		Normetanephrine	
	Concentration added (μg/L)	Recovery (%)	Concentration added (μg/L)	Recovery (%)
Level 1	985	110	974	97
Level 2	420	102	407	92
Level 3	142	94	97	94

detector. Ten replicates of a blank sample were analysed in a single run to determine the lower detection limit. The lower detection limit for MN was 3.3 μg/L (3 SD of the blank sample).

DISCUSSION

In surveying the data obtained from the laboratory evaluation of the Bio-Rad Urinary Metanephrines Reagent Kit, the following conclusions could be drawn. The reproducibility data (Table I), linearity data (Figs. 2 & 3), and recovery data (Table II) taken together indicate that the precision of an obtained test result for both urinary metanephrines is less than 5.6%. This method considerably improves the precision of the determination. This procedure eliminates the cumbersome chemical differentiation steps required for a separate determination of MN and NMN, and provides simultaneous analysis for NMN, MN and 3-methoxytyramine in a single chromatogram run in 20 minutes. Concentrations as low as 30 μg/L, well below the detection limits of many spectrophotometric procedures now in use, are readily measured with about the same investment in time and about half as many sample manipulations as required in previous methods. An experienced laboratory technician can prepare as many as half a dozen samples concurrently in less than two hours, including hydrolysis. This assay would not require discontinuation of the administered drugs that cause analytical interferences in the spectrophotometric assays. However, drugs known to cause physiological increases in catecholamines or metanephrines would still need consideration during the evaluation of phaeochromocytoma.

In conclusion, the performance characteristic of this kit was satisfactory and compares well with that reported by Bio- Rad.[12] We found the automated method to be versatile, user-friendly, efficient and once established, can be reliably performed by less experienced staff. Therefore, laboratories should consider automated sample processing and injection for high-performance liquid chromatography methods in the continual effort to improve diagnostic efficacy of biochemical tests for phaechromocytoma.

REFERENCES

1. Graham PE, Smythe GA, Edwards GA, Lazarus L. Laboratory diagnosis of phaeochromocytoma: which analytes should we measure? Ann Clin Biochem 1993; 30:129-34.
2. Sawka AM, Jaeschke R, Singh RJ, Young WF Jr. A comparison of biochemical tests for pheochromocytoma: measurement of fractionated plasma metanephrines compared with the combination of 24-hour urinary metanephrines and catecholamines. J Clin Endocrinol Metab 2003; 88:553-8.
3. Brain KL, Kay J, Shine B. Measurement of urinary metanephrines to screen for pheochromocytoma in an unselected hospital referral population. Clin Chem 2006; 52:2060-4.
4. Kudva YC, Sawka AM, Young WF Jr. Clinical review 164: The laboratory diagnosis of adrenal pheochromocytoma: the Mayo Clinic experience. J Clin Endocrinol Metab 2003; 88:4533-9.
5. Pisano JJ. A simple analysis for normetanephrine and metanephrine in urine. Clin Chim Acta 1960; 5:406-14.
6. Gupta RN, Price D, Keane PM. Modified Pisano method for estimating urinary metanephrines. Clin Chem 1973; 19:611-14.
7. Taylor RL, Singh RJ. Validation of liquid chromatography-tandem mass spectrometry method for analysis of urinary conjugated metanephrine and normetanephrine for screening of pheochromocytoma. Clin Chem 2002; 48:533-9.
8. Foti A, Kimura S, DeQuattro V, Lee D. Liquid-chromatographic measurement of catecholamines and metabolites in plasma and urine. Clin Chem 1987; 33:2209-13.
9. Shoup RE, Kissinger PT. Determination of urinary normetanephrine, metanephrine, and 3-methoxytyramine by liquid chromatography, with amperometric detection. Clin Chem 1977; 23:1268-74.
10. Flood JG, McComb RB. Urinary metanephrines as measured by liquid chromatography with an on-line post-column reaction detector. Clin Chem 1981; 27:1268-71.
11. Gupta RN. Improved sample preparation in determination of urinary metanephrines by liquid chromatography with electrochemical detection. Clin Chem1990; 36:538-40.
12. Bio-Rad Urinary Metanephrine by HPLC, automated method on ASPEC™ - instructions for use. Anaheim: Bio-Rad Laboratories, 2003: 1-26. ChB016551.