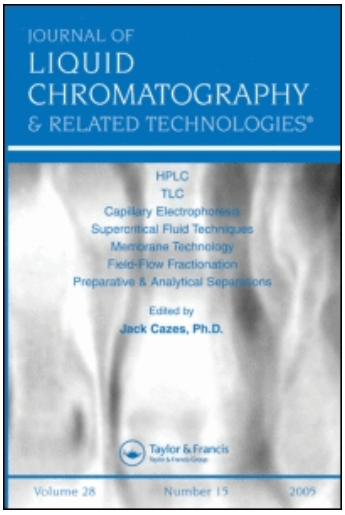

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A SIMPLIFIED AUTOMATED SAMPLE CLEANUP EXAMPLE: ANALYSIS OF URINE METANEPHRINES

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A SIMPLIFIED AUTOMATED SAMPLE CLEANUP EXAMPLE: ANALYSIS OF URINE METANEPHRINES

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ABSTRACT

Sample extraction and cleanup are important steps in many applications before performing the detection step. In this work we describe a simplified automated sample cleanup using only a sampler and a pump, which is suited for a small number of samples. The buffer continuously flows through a small column, which is regenerated and re-used. As an example, cleanup of urinary metanephrine is used before the HPLC step. Urine samples, in addition to the different buffers of equilibration, wash, and elution were placed on the sampler.

A peristaltic pump was used to deliver the sample and the buffers through a small column, about 200 μL of cationic resin, which was regenerated before each use, by the regeneration phosphate buffer. Sample recovery for metanephrines was 80-90% and the reproducibility was about 6%.

This type of automation is very simple and non-expensive. Furthermore, the same column is utilized for many samples

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decreasing, further, the cost of analysis and improving the precision by avoiding the between-column variability.

INTRODUCTION

Sample cleanup is a very important step in the analysis of compounds present in low concentration among numerous interfering substances. It is common in the analysis of drugs and many metabolites present in biological fluids. Different approaches are used to achieve sample cleanup. However, solid-phase extraction is the most common of these methods. This can be performed manually, on-column (in the loop), semi-automated, and automated using different kinds of robotics. In the majority of these techniques a separate column is used for each sample leading to a high cost per test.

We used, here, a different approach, in which the column is regenerated and used for multiple samples, while the buffers are pushed through the column continuously. The instrumentation is simplified considerably by using only a pump and sampler. This approach leads to decrease in the cost per analysis and better precision by the elimination of the differences between columns.

We report here analysis of urinary metanephrines (metanephrines and normetanephrines) as an example to test this approach. These compounds arise from the metabolism of the catecholamines. Usually, they present in low concentration, less than 1 μg/mL among many interfering compounds. However, these metanephrines increase 5-50 fold in the urine of patients with the special tumors known as pheochromocytoma and neuroblastoma.(1,2) These tumors lead to hypertension.

Several HPLC methods have been described for the analysis of metanephrine.(3-5) However, the most common method used in routine laboratories is the colorimetric method of Pisano(6,7), which converts metanephrines to vanillin. In all these methods, extensive sample extraction is necessary.

Sample cleanup is achieved by passing the urine on a wet cationic resin (~200 μL of Amberlite-GC50), which is regenerated automatically before each use followed by elution of the metanephrines in a high ionic strength buffer. The collected fraction is injected on a C_{18} Column (HPLC) with detection of the native fluorescence of the metanephrines in the ultraviolet range of the spectrum. The combinations of an ion exchange mini-column, the analytical C_{18} column, and the native fluorescence all lead to clean chromatograms.

EXPERIMENTAL

Reagents

1. Amberlite Resin CG-50 AR Grade 100-200 mesh (Sigma Chemicals, St. Louis, MO) was prepared as described by Pisano.(6)

2. Cleanup Column: About 150 µL of wet prepared cationic resin in step 1 (Amberlite GC50) was packed in a tip of disposable 250 µL pipette. Several columns were prepared at a time and stored in the generation buffer. Each batch was checked before use.

3. Generation Buffer: 2.0 g NaH_2PO_4, 0.6 g Na_2HPO_4 and 2.0 mg sodium azide in 100 mL water adjusted to pH 6.1.

4. Elution Buffer: 6.0 g Na_2HPO_4, 0.4 g NaH_2PO_4 and 15 g of KCL in 100 mL water adjusted to pH 7.4.

5. Mobile Phase: Phosphoric acid, 1.0 mL was diluted in 1000 mL water.

6. Metanephrine Stock Standard: Metanephrine,10 mg and normetanephrine,10 mg were dissolved in 100 mL water.

Procedure

Urine, 1 mL was acidified with 100 µL of 2N HCL and hydrolyzed at 95°C for 20 min. Hydrolyzed urine, 50 µL was diluted with 2.0 mL generation buffer. The standard was placed in cup 1 on the sampler followed with 2.0 mL generation buffer in cup 2, 2.0 mL of elution buffer in cup 3, and 2.0 mL of generation buffer in cup 4. This was repeated for each urine sample. The two effluents of the elution buffer and the second generation buffer were collected and mixed as they dripped from the column into a large cup placed on the sampler. An aliquot of 20 µL was injected on the HPLC column.

Instrumentation

The two main components for the analysis of metanephrines were:

Clean Up Instrument

1. A peristaltic pump (Technicon Corporation, Tarrytown, NY) to push the buffers at flow rate of 0.4 ml/min, and 2. Sampler (Technicon Corporation) to select the buffers and also to accept the purified fractions as illustrated in Fig 1 and Fig 2.

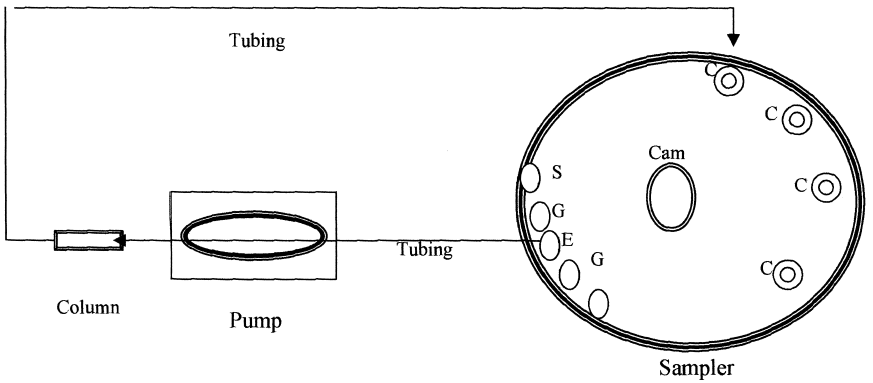

Figure 1. Schematic of the instrument: S, sample cup; G, generation buffer (wash step); E, elution buffer; G, generation buffer (equilibrium step); and C, collection cup.

HPLC Instrument

A Model 110 A pump (Beckman Instruments, Palo Alto, CA) was set at flow rate of 1 mL/ min to deliver the buffer through an analytical column C_{18}, 5 µm, Microsorb (Varian Analytical Instruments, Walnut Creek, CA). The detector

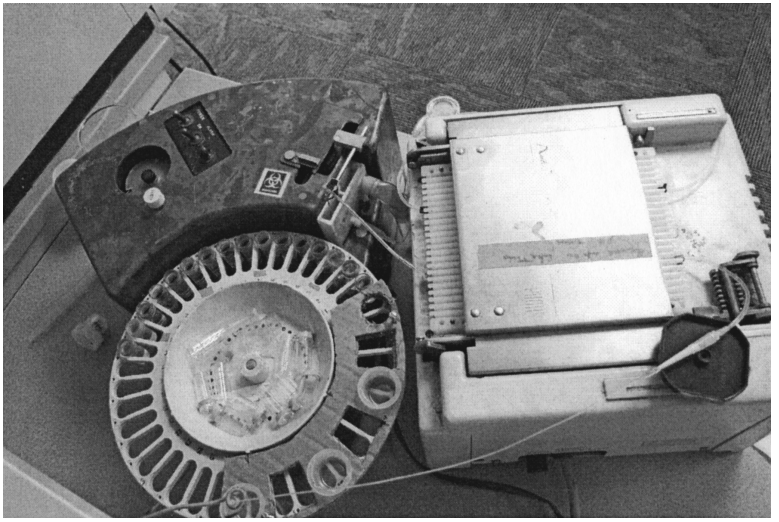

Figure 2. Picture of the instrument.

RF-10AXL (Shimadzu, Columbia, MD) was set at Excitation 280 nm, Emission 315 nm.

RESULTS AND DISCUSSION

Urine contains both metanephrines and normetanephrines. These metabolites are excreted as both free and conjugated (with sulfate and glucuronides). Acid hydrolysis is an important step for releasing the metanephrines from these groups for subsequent analysis.

The extraction instrument is composed of two parts, a sampler and a pump. The peristaltic pump continuously pushes the different buffers through the column. The probe on the sampler dips continuously into each cup for 2 min, actuated by a cam. The main feature of this work is simplifying the cleanup instrumentation by manipulating the sampler to perform multiple functions: sample delivery, fraction collector, and buffer selector. Thus, it eliminates the need for many additional parts. This is accomplished by placing the cups of wash (with the generation buffer), elution, and re-generation (for equilibration) buffers after each cup of urine, Fig 1. This order is repeated for every urine sample.

Because of the multi-function of the sampler, the test has to be well optimized in order to elute and collect the desired fractions at the appropriate time. Unfortunately, optimizing the purification step is based on trial and error. The combination of flow rate, cam speed, column dimensions, and concentration of the different buffers all have to be optimized and synchronized for each test. The desired fractions eluted from the column are collected in empty cups on the same sampler. The speed of the analyzer is 7.5 samples /hr. Adding another sampler to the pump doubles the speed. Cups, which contain the eluted metanephrine fractions, are injected manually on the HPLC (C_{18}) column.

Figure 3 illustrates the effect of sample cleanup on the analysis of urine metanephrines. The extracted standard is represented in Fig 3-A. In the absence of sample cleanup the peaks are obscure and cannot be detected, Fig 3-C. Furthermore, several late peaks keep on eluting, increasing the analysis time. The combination of cleanup step, the analytical C_{18} column, and native fluorescence all lead to clean chromatograms, Fig 3-B. The linearity was tested between 0.5-10 mg/L, Fig 4 ($r = 0.991$ for normetanephrine and 0.993 for metanephrine). The recovery for both metanephrine and normetanephrine were between 80-90%, depending on the column. The reproducibility (CV) of the analysis of normetanephrine was 5.1% and metanephrine was 5.8% (n=8).

The suitability of this test for detecting pheochromocytoma was tested by analyzing seven patients with proven pheochromocytoma (by tissue pathology). These patients showed a ten times elevation of normetanephrine compared to 30

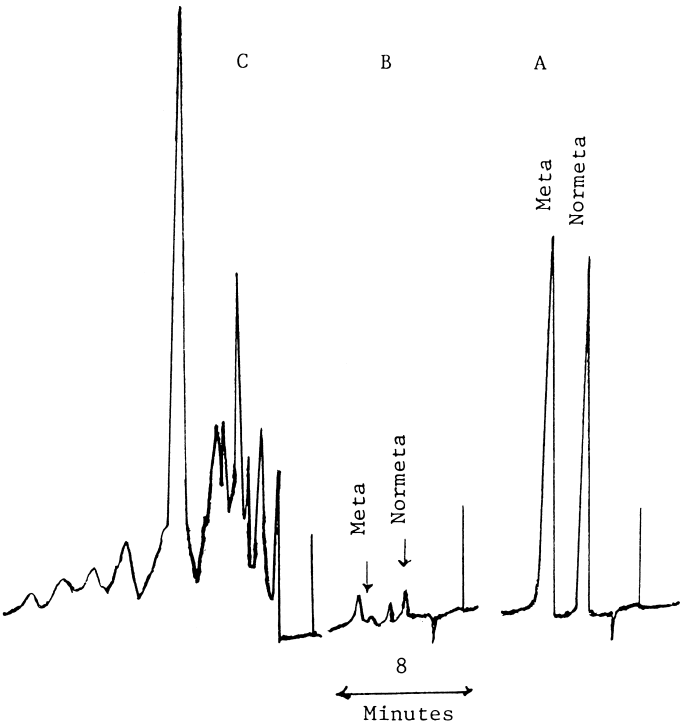

Figure 3. Chromatogram of: A, standard (4.5 mg/L of each normetanephrine and metanephrine); B, urine after cleanup by the instrument; and C, the urine before the cleanup.

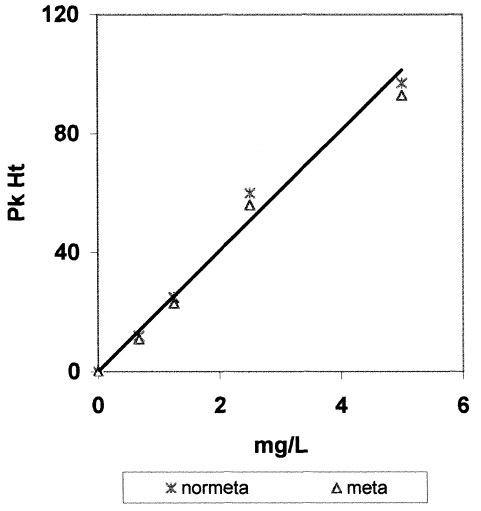

Figure 4. The linearity of the assay.

Table 1. Metanephrines in Normal Individuals vs. Patient with Pheochromocytoma (mg/L)

	n	Normetanephrine	Metanephrine
Normal	30	0.45 ± 0.25	0.1± 0.1
Pheochromocytoma	7	4.8	0.2
		(0.7–12.4)	(0–0.7)

Reference range (total): 0-1.3 mg/g creatinine.

normal individuals, Table 1. These values are comparable to those in the literature.(1) Another additional patient with pheochromocytoma had unusually very high normetanephrine of 237 mg/24 hr (over 200 times the upper normal range). His tumor was located in the kidney and weighed 2.3 kg (about 20 times the average tumor).

In addition to the metanephrines, both epinephrine and norepinephrine are cleaned and elute, too by the Amberlite column. However, they require addition of ion pairing reagent to the HPLC solvent for optimum separation. Tests which can be analyzed with small sample-load and with sensitive detectors, such as fluorescence, electrochemical, or isotopic can be cleaned by this instrument. We have also tried performing homovanillic acid after clean up with a C_{18} mini-column and using electrochemical detection. The recovery was also satisfactory, about 80%.

Advantages of this type of cleanup are full automation, instrument simplicity (less expensive, uses the same column), better precision and flexibility for different tests. This instrument is very simple; since it does not require switching valves of the different buffers, separate fraction collector, or computerization.

Native fluorescence detection for metanephrines has not been as common as the electrochemical detection.(4,5) It does not require the addition of further chemical reactions to induce fluorescence. Previously, we have shown that the new generation of fluorescence detectors have much improved sensitivity and stability, making these instruments more rugged and more practical for routine work when compared to the electrochemical detector.(8)

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