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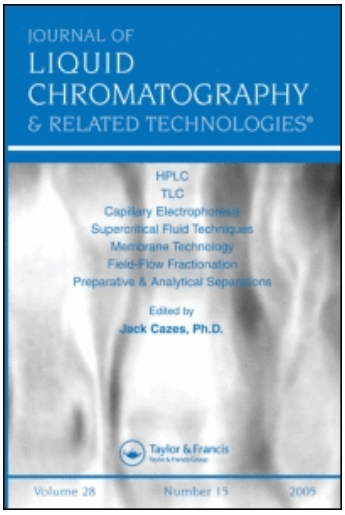

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Differential Analysis of Blood Urea Using Combined Automated Ultrafiltration and Solid Phase Extraction in On-Line Series

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DIFFERENTIAL ANALYSIS OF BLOOD UREA USING COMBINED AUTOMATED ULTRAFILTRATION AND SOLID PHASE EXTRACTION IN ON-LINE SERIES

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ABSTRACT

Solid phase extraction (SPE) is an efficient pretreatment method to separate a compound of interest in complicated matrix such as body fluid, food, or in soil. Ultrafiltration is also a suitable pretreatment method to remove higher molecular weight compounds such as protein in body fluid. The latter method is also useful for differential analysis of free type from protein-bound type compound in body fluid. Free type compound after ultrafiltration will exist in filtrate. Previously ultrafiltration and SPE were carried out off-line and the former was done manually, which was troublesome for routine analysis. In this paper these pretreatment methods were successfully on-line combined and controlled by computer first by the author. Unfortunately this kind of equipment is not currently available in the market yet due to patent restriction.

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INTRODUCTION

Several pretreatment methods for separating admixtures in complicated matrix such as body fluids, soil, food, and others have been reported so far.[1,2] Ultrafiltration, dialysis, supercritical fluid extraction, solid phase extraction, (SPE) are currently available and other methods are under research for pretreatment method.[1,2] Supercritical fluid extraction has a restriction to hydrophobic compound extraction. Other methods have no such restriction. Supercritical fluid extraction has a superiority that extraction procedure is done by gas, thus it is unnecessary evaporation or condensation after extraction, which is so often required in liquid-liquid extraction or SPE extraction. This procedure so often resulted in lower recovery due to unsatisfactory trapping or thermal degradation. Shintani firstly reported for blood urea analysis using a cation exchange resin SPE.[3,4] At that time when he published papers, only manual type SPE combined with off-line ultrafiltration methods were available and used for differential analysis of bound urea from free urea, as well as free ammonia in blood.[3]

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Recently the automated on-line SPE equipment, BenchMate, from Varian Co. Ltd., became available, but even now it is not available in the market of computer contolled on-line ultrafiltration equipment with HPLC. Therefore, the author invented the combined on-line automated SPE ultrafiltrator and HPLC for differential analysis in routine work. This method can be applicable for differentiation of "free" from "bound" urea. Accurate free urea analysis is essential in clinical tests to attain precise uremia diagnosis. Combination of on-line automated SPE and ultrafiltrator was successfully attained using the author's invented software, of which the patent is now under application. Unfortunately, the concrete content of the patent is not public right now due to it being under application to Japan patent agency. Soon in the future this patent can be opened and the author can release the approval of patent use for manufacturer's benefit. Using this combined equipment, differential analysis of blood urea can be attained more easily in routine work. This equipment is not available on the market in the reader's hand and the author hopes it will be available soon after manufacturer's marketing research, especially in health care facilities, pharmaceutical companies, and medical device companies.

MATERIALS

Urea used was from Wakoh Co. Ltd. Blood and urine were sampled from the author before breakfast. Serum was prepared from blood by ultrafiltration. Other reagents used were special or HPLC grade.

EXPERIMENTAL

SPE and HPLC Conditions

SPE equipment was BenchMate® from Varian Co. Ltd. SPE column was Bond Elut® SCX (strong cation exchange resin column with H type from Varian). Resin weight and resin volume of Bond Elut® SCX were 500 mg and 2.8 mL, respectively. Conditioning was done by 3 mL of methanol followed by 3 mL of water at the flow rate of 3 mL/min. Rinsing was done by 1 mL of water at the flow rate of 3 mL/min. Elution was done by 4 mL of 5% phosphoric acid solution at the flow rate of 1 mL/min. Conditioning, rinsing, and elution were carried out under vacuum identical to the previous papers.[5-7]

HPLC after SPE treatment was carried out as follows: Column was MCI GEL CK 08S®(polymer base strong cation exchange resin column with Na type, 4.6 X 150 mm) from Mitsubishikasei Co. Ltd. Eluent was 1mM HCl solution and flow rate was 1mL/min. Detection was by UV at 200 nm and applied sample volume to HPLC was 20 μL. The column temperature was 35°C. Pump and detector were PU-980® and PU-970® from Nihonbunko Co. Ltd., respectively.

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Ultrafiltration and Combination of SPE, HPLC, and Ultrafiltration

Native blood and denatured blood with acid were centrifuged with ultrafiltrator of H-1300® from Kokusan Co. Ltd. at 13,000 rpm (10,000 g) for 60 minutes. The supernatant was sampled by controlling with computer installed software, which is under application. Supernatant sample was for free type urea analysis.

Automated ultrafiltrator and HPLC was on-line combined and controlled with a computer as mentioned in advance.

Membrane for ultrafiltration is Centrifree® from Amicon Co. Ltd., made of cellulose with cut-off molecular weight of 10,000 daltons, indicating direct injection of supernatant of ultrafiltrate may deteriorate HPLC column with less than 10,000 daltons peptides and other compounds with identical molecular weight; prior to HPLC application, and after ultrafitlation procedure, SPE treatment is required. Ultrafiltration is for differential analysis of "free" from "bound" urea. Native blood and denatured blood are for analysis of free and total urea, respectively.

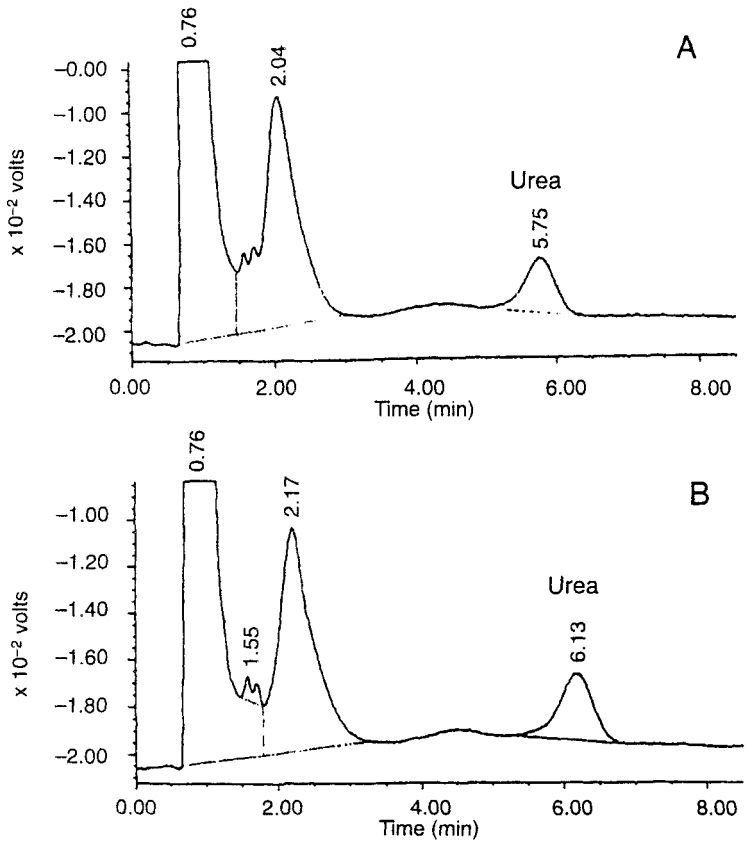

Figure 1. HPLC chromatograms after on-line automated SPE and ultrafiltration in series (a) Blank serum; (b) Spiked serum with urea at the concentration of 0.1 mg/mL.

RESULTS AND DISCUSSION

The HPLC chromatograms of supernatant of ultrafiltration procedure after SPE treatment is presented in Figure 1. The on-line ultrafiltrator, SPE and HPLC were combined in series in this order and controlled with computer. Figure 1 (a) presents a chromatogram of serum blank and Figure 1 (b) presents that of spiked urea to serum at the concentration of 0.1 mg/mL. As shown in Figure 1, chromatograms of blood supernatant treated with ultrafiltration and SPE indicated that urea peak was free from blood admixtures and blood urea was satisfactory recovered from strong cation exchange SPE column.

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Elution time of urea of strong cation exchange analytical column was around 6 min. The recovery rate of spiked urea was 101.9%, RSD=2.2% (n=4). Standard addition curve indicated that the endogenous urea amount was 0.82 mg/mL obtained from the cross point to the horizontal line (x-line).

The total urea amount obtained from denatured ultrafiltrate with acid was around 1% greater (n=4) than normal blood ultrafiltrate, indicating bound type urea was around 1%. It was reported that blood urea was not significant and mostly bound to albumin in blood.

Ammonia in urine was around 10% (n=5) of urea in urine, thus separation by strong cation exchange HPLC is essential. As is being done in current health care facilities using autoanalyzer with colorimetry and using the indophenol method, combined with visible detection as ammonium or ion selective ammonium method, using immobilized urease attached to an electrode, differential analysis of urea from ammonia cannot be attained and be determined as ammonia as a whole. Thus, separation with cation exchange HPLC is required in health care facilities in the future for precise analysis of free urea in blood, for precise diagnosis of uremia.

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CONCLUSION

For the differential analysis of blood urea analysis, it was found that an on-line automatically controlled method of automated SPE and automated ultrafiltlator in series was essential. This is because free type urea accumulated in blood may promote uremia syndrome; therefore, precise determination of free urea is more important than total blood urea analysis, as blood urea nitrogen (BUN) currently done in health care facilities.

Because increased blood urea, which should be excreted into urine if kidney function is normal, can be accumulated in blood and the accumulated blood urea may cause feedback inhibition of urea cycle, which is the final cycle of protein digestion, indicating protein metabolites will not function well due to accumulated blood urea.

The on-line computer controlled ultrafiltrator, SPE and HPLC in series is useful for routine differential analysis. As this equipment is successfully prepared by the author, it can be supplied in the market soon in the future. This equipment will be essential for health care facilities for clinical testing in routine work.

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