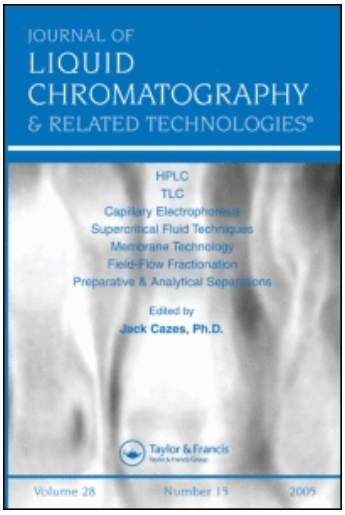

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Solid Phase Extraction (SPE) of Blood Urea Compared with Liquid-Liquid Extraction Regarding Artifact Formation

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SOLID PHASE EXTRACTION (SPE) OF BLOOD UREA COMPARED WITH LIQUID-LIQUID EXTRACTION REGARDING ARTIFACT FORMATION

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ABSTRACT

Solid phase extraction (SPE) of blood urea is described. Differential analysis of free type and bound type blood urea analysis for diagnosis is important because free type urea is more essential causing symptom of uremia. The differential analysis is attained by ultrafiltration by centrifugation using a cellulose membrane with cut-off molecular weight of 10,000 daltons from native and acidified blood. Compared with SPE, liquid-liquid extraction is more problematic due to the formation of artifacts during extraction and condensation by evaporation. Artifacts were observed in solvent extraction as a result of reactions between the extraction solvent and the target compounds.

INTRODUCTION

When a known compound is to be extracted by a solvent, the property of solubility and polarity of a target compound must be considered. When an unknown compound is to be extracted, systematic studies are prepared to extract the compound with different solvents and analyze the biological as well as physiological activities of each extraction fraction to examine in what fractions physiologically-active compounds were extracted. Thereafter, further separation using a proper method such as chromatography will be considered. This is a normal procedure when separating an unknown compound.

It is a so-called bioassay to examine in what fraction the physiologically-active compounds were extracted. Based on the author's experiences, the author intends to stress that in addition to the solvent properties there is another factor to be considered when selecting an appropriate solvent for extraction due to the artifact formation during solvent extraction.

As another subject, the author wishes to present blood urea analysis by using solid phase extraction (SPE) because of no previous studies of its superiority to liquid-liquid extraction procedure. There has been many papers describing chromatograpy of an immobilized enzyme, post column reaction or column switching, which required complicated procedure, set-up systems and troublesome technique[1,2]. This is because the separation of blood urea from other blood components is so difficult, therefore researchers designed complicated techniques. However, the complicated procedures are not easily applicable to routine clinical analysis. In this paper the author will present the simple technique of blood urea analysis using SPE instead of the complicated chromatographic separation.

MATERIALS AND METHOD

Toxic compounds in polyurethane (PU) were studied and the PUs used are identical to these reported previously [3].

HPLC analysis method for blood urea is as follows: Mitsubishi Kasei MCI^{R} CK 08S strong cation exchange column (SO_3H type, 4.6X 150 mm) was used. Column capacity and particle size were as follows: more than 1.9 meq/ml and 11-14 um. The material is SDB polymer base (degree of crosslinking of DVB is 8%). Other information on MCI Gel CK/CPK series for strong cation exchange columns are available from Mitsubushi Kasei Co.Ltd. The eluent is 1.5 mM HCl aqueous solution and urea is detected by ultraviolet at the wavelength of 210 nm. Flow rate is 2 ml/min. Retention time of blood urea peak is 24 min. HPLC apparatus is SP-8750 from Spectra-Physics.

Liquid-liquid extraction using methanol or acetone is identical to that reported previously[3].

SPE of blood urea is as follows: Blood is acidified at pH 3 with HCl or used as is. These were applied for ultrafiltration by centrifugation at 4,000 rpm using a Centricon^{R} (cut-off molecular weight 10,000 daltons) supplied by Amicon and the centrifugated

solution was acidified to pH3 with HCl. These were applied to the strong cation exchange column (H type) of Bond Elut^R SCX (500 mg of resin weight and 0.6 ml of void volume) supplied by Analytichem (Harbor City, CA, USA). The column was conditioned with 2 ml of methanol followed by 2ml of 0.1M HCl aqueous solution, which is a normal manner previously described[4]. One ml of blood was applied to the conditioned SCX column at an application flow rate of 0.3 ml/min and washed with 2 ml of water. The retained urea on SCX column was eluted with 2 ml of 1M HCl aqueous solution at the flow rate of 0.3 ml/min. Conditioning and elution in SPE were carried out using a Model AP-115 AN vacuum pump supplied by Iwaki (Tokyo, Japan)[4].

RESULTS AND DISCUSSION

1 Artifact formation during extraction

While studying the extraction of toxic compounds and the physiological activity of the extracts (such as carcinogenic, mutagenic, cytotoxic, teratogenic compounds), the author found that the physiological and biological activities and recovery rate of an extract vary depending on the extraction solvents. These phenomena may not seem particularly surprising but it should be stressed here that these are not always caused by the difference in chemical as well as physical properties and polarities of extraction solvents.

For example, a medical device such as an artificial dialyzer was extracted with methanol or acetone, and each extract is tested for mutagenicity Ames test with or without S9 Mix of metabolic activity enzyme (Table I)[5]. Acetone indicated a greater quantity of extracted substances than methanol, but the substances extracted with acetone indicated a lower mutagenicity (Table I). Therefore, the researcher may speculate that mutagenic compounds are more favorably extracted with methanol rather than acetone. Cunningham et al[6] stated that the solvent, especially methanol, reacts with the target compound such as amine compounds and produced physiologically more active and more toxic compounds, which resulted in an artifact formation. If the researcher overlooks this kind of artifact formation, he may have an incorrect decision and misunderstanding for his results. The results reported by Cunningham et al are

TABLE I Mutagenicity test of irradiated and non-irradiated polyurethane extracts

Solvent	Extracted Amount (g)	Number of Mutant at 5000 μg/plate in the Absence of S9Mix*	
		non-irradiation	10Mrad
MeOH	4.97	173	1014
Acetone	8.10	134	249

*Salmonella typhimurium TA 100 was used.
Extraction was from 46 g of each polyurethane.

briefly outlined below followed by the methods for solving or preventing the artifact formation problems, some of which were based on the author's own experience.

During extraction with methanol or a vacuum evaporation for condensation process Cunningham et al observe that some aromatic amine compounds undergo a Mannich reaction with formaldehyde from methanol[6]. During these reaction, an artifact of dimer or oligomer compounds was produced. These artifact compounds indicated a greater mutagenicity more than 5 times than the original target compound[6]. The author also confirmed the formation of similar oligomer artifacts when extracting aromatic amine compound with methanol from polyurethane. Aromatic amine compound in this case is 4,4'-methylenedianiline (MDA) from PU and the dimer or oligomer linked at R-NHCH2NH-R was produced. Formation of such oligomer artifacts is prevented by replacing methanol with ethanol.

Furthermore, the liquid-liquid extraction process is replaced by SPE in order to prevent artifact formation and increase recovery rate of target compound[3]. This is because the former requires vacuum evaporation/concentration and allows prolonged contact of the target compound with the extraction solvent. Additionally, it is a well-known fact that compounds were lost without successfully trapping during a vacuum

evaporation/concentration process, thus reducing the recovery rate and causing thermal decomposition[3].

Cunningham et al also observe the formation of acetylated artifact when using ethylacetate as an extraction solvent. This is because the compounds with hydroxyl or amino functional group will be acetylated during extraction and reduce the recovery rate of the target compounds with hydroxyl and/or amino functional groups. These acetylated compounds also indicated a greater toxicity, therefore they warn that the results may lead to the misunderstanding that compounds appearing to be strongly mutagenic are collected[6].

II Blood urea analysis using SPE combined with ion exchange chromatography

Blood urea analysis using HPLC has already been reported. However, most of them are complicated set-up system such as using an immobilized enzyme column prior or posterior to the analytical column, post-column calorimetry or florescence reaction method, pre-column calorimetry or florescence reaction method or column switching method[1,2]. They required complicated technique, thus they are not favorably applicable to the routine clinical laboratory.

SPE for blood urea has not been reported so far, therefore the author intended to prevent blood components with SPE in place of chromatographic separation in addition to the differential analysis of endogenous ammonia and urea. Some part of blood urea existed in combination with blood protein, therefore ultrafiltration using the centrifugation at 4,000 rpm is thought to be most appropriate for differential analysis of free type from protein-bound type of blood urea.

By acidifying the blood, urea is totally isolated from blood protein due to denaturation of blood protein, therefore the differential analysis of free type and free plus bound type is attainable. The analysis of free type of blood urea is more essential for the purpose of diagnosis of patient's status of disease. Current clinical test is carried out using a selective ammonia electrode. By this method, the differential analysis of endogenous ammonia and urea is not attainable, thus the selective analysis of endogenous urea analysis is required.

For SPE procedure of blood urea, the centrifuged solution is applied to Bond Elut^{R} SCX column and eluted with 1 M HCl as mentioned in the experimental section.

For HPLC analysis, urea elution will vary depending on the concentration of HCl aqueous solution used for the eluent as well as the retention time and separation of blood urea from blood components. As the results, 1.5 mM HCl aqueous solution for the eluent and 2 ml/min flow rate were found to be appropriate in the current experiment. This result is dependent on the difference of blood or other factors such as the amount of components in blood. Thus the reader can select between 1-2 mM HCl for the eluent at the flow rate of 2-3 ml/min. The author did not believe current result in this paper was most appropriate and required modification depending on sample blood. The selection of columns for blood urea analysis was studied and compared the current and conventional ion exchange column with the ion chromatography column with less ion exchange capacity. The former was found to be superior to the latter in terms of urea separation from blood components[7].

Blood urea peak was not interfered with blood components in my experiment as shown in Figure 1. In order to confirm urea peak was not overlapped and a single peak, photodiodearray detection with multiple wavelength for three dimension chromatogram was used. This result indicated urea peak was single and pure.

By using the combination of SPE and HPLC methods, complicated HPLC analytical procedure is not required. Routine and differential analysis (free and bound type) is easily attainable. Blood urea is free from blood components using SPE in place of chromatographic separation as shown in Figure 1. The average recovery rate of blood urea following this method of SPE is 98% (n=10), which is satisfactory.

Current SPE procedure was carried out manually, but the author thinks the combination of autoinjector, automated ultrafiltration using centrifugation, the automated SPE, and HPLC in series will lead to the development of a laboratory clinical test device that will be more convenient than the current clinical test method using an ammonia-selective electrode. The main reason is due to the capability of differential analysis of free and total urea amount in blood in addition to more precious, selective and sensitive than current method using the ammonia-selective electrode. In terms of automation, for example, when combining ASTED^R (Automated Sequential Trace Enrichment of Dialysates) and ASPEC^R (Automatic Sample Preparation with Extraction Columns) from Emuesu Co. Ltd were used combined with autoinjector and HPLC, automated equipment for differential analysis of free and bound type blood urea will be attainable for clinical laboratory routine analysis.

Moreover, the necessity of differential analysis is not always limited to the determination of blood urea. Blood guanidines, calcium in milk as an example are also

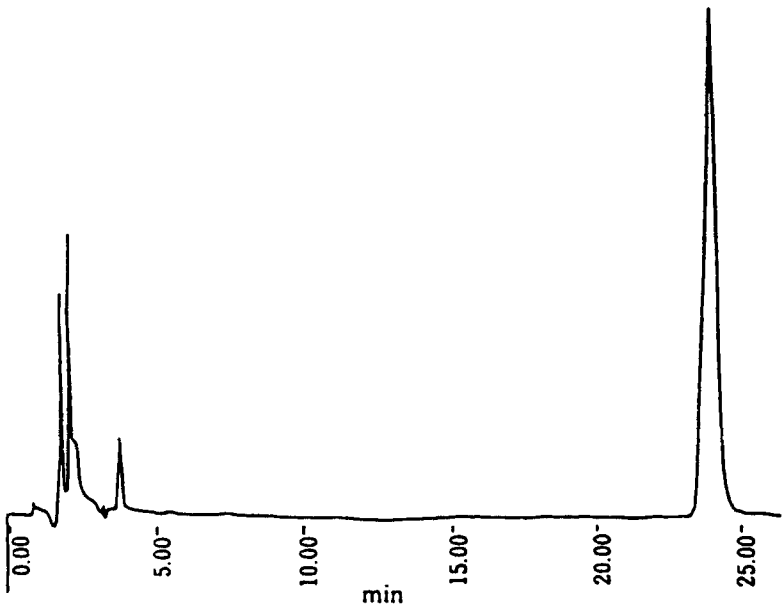

FIGURE I HPLC chromatogram of blood urea after SPE treatment by SCX column

The peak eluted at around 24 min is blood urea.

required. Therefore, the current method as well as an automated procedure may be applicable for the determination of other components in clinical laboratory as well as other industries.

CONCLUSION

Analytical chemists are not well informed on artifact formation during solvent extraction by reacting the extraction solvent with the target compound, which may lead to incorrect decision and misunderstanding for their results. In order to avoid these, the author stresses the necessity of taking into consideration the factors other than solvent properties and polarities when selecting appropriate extraction solvents and the necessity replacing liquid-liquid extraction with SPE.

SPE is superior to liquid-liquid extraction. By using SPE, routine and differential analysis (free and bound type) of blood urea is easily attainable and blood urea is free from blood components by using SPE.

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