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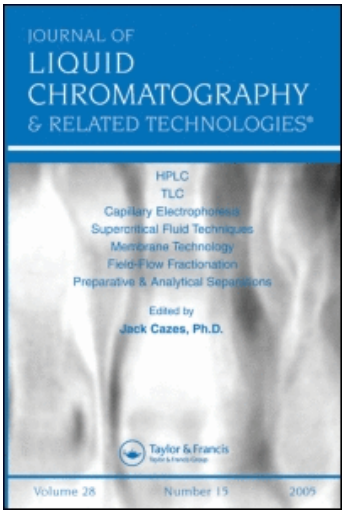

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Selective Analysis of Compounds in Body Fluids and How to Avoid Artifact Formation

H. Shintani[a]
[a] National Institute of Health Sciences Ministry of Health and Welfare, Kamiyoga Setagaya, Tokyo, Japan

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SELECTIVE ANALYSIS OF COMPOUNDS IN BODY FLUIDS AND HOW TO AVOID ARTIFACT FORMATION

H. Shintani

National Institute of Health Sciences
Ministry of Health and Welfare
1-18-1, Kamiyoga
Setagaya, Tokyo, Japan

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ABSTRACT

Elution of starting compounds as well as artifact compounds from medical-use polymer material was studied. Artifact formation during medical polymer fabrication, as well as the artifact formation during contact of medical polymer with body fluids, has been so often overlooked. The analysis of starting compound residue in medical-use polymer has previously only been discussed. In this paper, the author studied the importance of recognizing the artifact formation to attain reproducible recovery of compound of interest from complicated matrix; an appropriate pretreatment method to make compounds of interest free from admixtures in body fluids and to attain reproducible HPLC analysis. Artifact compounds must be identified prior to determination. In order to attain reproducible analysis in complicated matrix, appropriate recovery and isolation methods as a pretreatment method, are required. Chemical structure identification of newly found toxic compounds from dental material was carried out by high performance liquid chromatography (HPLC) - mass spectrometry (MS)-MS.

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Determination of these compounds to saliva was by HPLC combined with solid phase extraction. Selection of appropriate eluent was studied. The eluent selected was opposite from the conventionally speculated eluent, but the selected eluent has reproducibility and a scientific rationale.

INTRODUCTION

Solid phase extraction (SPE) using packed columns combined with high performance liquid chromatography (HPLC) for analysis of several toxic compounds in blood was described. Residual dental monomer analysis with HPLC was already reported, but these papers are restricted to starting compound analysis.[1-5]

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The poly methylmethacylate (PMMA) is widely used as the composite resin for the dental plate. For PMMA fabrication, benzoylperoxide (BPO) and N,N-dimethyl *p*-toluidine (DMPT) were added as polymerization initiator and the stimulator, respectively, for PMMA fabrication. If insufficiently polymerized, MMA monomer, DMPT and BPO may exhibit a potential residue.[1-5] Residue of MMA was especially significant at around 1-2% (10,000-20,000 ppm). When considering that 1-2% of toxic compound of MMA will be eluted to patients during and after dental treatment for a prolonged period of the patient's life, this amount will be larger due to degradation during use. Thus the elution amount from dental plate prior to treatment and during fabrication must be diminished for the patient's benefit.

As additional information, newly found compounds, which have not been reported so far, were identified using HPLC-mass spectrometry (MS)-MS. One of the newly found toxic compounds was 2,3-epoxide compound of DMPT. Epoxide compound was quite reactive to DNA, so is most probably mutagenic and carcinogenic as is ethylene oxide. BPO, which was also a quite reactive compound, was converted to benzoic acid (BA) in a few seconds when contacting with DMPT or body fluids such as blood or saliva. Therefore, BA analysis has identical meaning to that of BPO determination in body fluids. BA was not originally utilized for PMMA fabrication, indicating BA is a sort of artifact from BPO when contacting with body fluids or contacting with DMPT during fabrication. BPO is a quite reactive compound and both BPO and BA are also cytotoxic compounds.[5]

For determining cytotoxicity, serum was added in a cell culture medium, therefore BPO transformed to BA immediately, which was first confirmed by the author. Therefore, the cytotoxicity data already reported as BPO was not BPO data, but BA data. The cytotoxicity data of IC50 (μg/mL) of BA and BPO

using Balb 3T3 cell was 28.7 and 22, indicating both are almost identical because BPO data was originally BA data with some analytical deviation mostly due to difference of culture medium lot.

In order to evaluate, statistically, the risk factor to the recipient exposed to these compounds from dental material for long life periods, the author initially studied reliability and reproducibility of the method. Thereafter, it was quantitatively determined that residual amounts in composite resin using serum extraction combined with SPE,[1-5] MMA and BPO are unstable upon heating; therefore, HPLC is considered to be superior to GLC. Selection of an appropriate column for dental material analysis was already studied.[3] Determination was carried out by HPLC combined with SPE using C_{18} columns in both cases. The comparison of several eluents of these compounds from C_{18} SPE column was studied. The comparison of SPE and liquid-liquid extraction in terms of recovery efficiency was also studied.

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MATERIALS

Blood and saliva used are sampled from the author. Ten mL of saliva from the author was sampled everyday before breakfast. Standard saliva is not available in the market. Therefore, depending on source of saliva, determination data presented in this paper may differ, but not significantly because of difference of saliva constituents such as protein, etc. Additionally, for example, the author has already been treated by a dentist, so patient history must also be considered. Fortunately, no compound elution was observed from the author's blank saliva. Most of the chemicals, except for compounds synthesized by the author in this paper, were available in the market.

METHODS

MMA, DMPT and BPO Analysis

The column for MMA, DMPT, and BPO analysis was Capcell Pak® C-18 SG-120 from Siseido Co. in Tokyo. This column was completely endocapped with silicone coating. Residual silica effect need not be of concern. The eluent was a mixed solution of water and acetonitrile at a ratio of 1/1 (v/v), without any addition of common ion, to the eluent for DMPT analysis due to a completely endocapped column. The flow rate was 1.2 mL/min. Detection was by UV at 235 nm.

BA Analysis

BA analysis was as follows: a Capcell Pak® C_{18} AG-120 column was used with an eluent of a mixture of acidified aqueous solution of water and acetonitrile at a ratio of 4/1 (v/v) adjusted to pH 3 with acetic acid. Acidification is for BA depression, otherwise BA will be eluted before void volume. Detection was by UV at 235 nm. The rest of the procedure was identical to MMA, DMPT, and BPO analysis.

Newly Found Toxic Compound Analysis

Methanol extract of dental material, fabricated at room temperature of Yunifast® from GC Co., Tokyo, was used for unidentified compound analysis. Using the gradient elution of HPLC shown in the following section, many compounds including MMA, DMPT, and BPO were eluted. Total peaks eluted were not completely identified, but the identified compounds were shown as follows: aniline, N-methyl *p*-toluidine, *p*-toluidine, BA, 3-carboxy 4-N-methyl amino toluene, 2-carboxy 4-N-methyl amino toluene, 2-carboxy 4-amino toluene (3-amino 6-methylbenzoic acid), 3-carboxy 4-amino toluene (2-amino 5-methylbenzoic acid), 2-hydroxy DMPT, 3-hydroxy DMPT, 2,3-epoxy DMPT, and *o* and *p*-N-methyl amino benzoic acid were identified.

In this paper 2 or 3-hydroxy DMPT and 2,3-epoxy DMPT was discussed in detail for a pretreatment and reproducible determination method.

The hydroxide derivatives of DMPT were synthesized by the author as follows: Each five grams of 2-amino- 5 methyl-phenol or 3-amino- 6 methyl phenol, 10 grams of methyl iodide (methylating reagent), and 5 grams of potassium hydroxide were refluxed for 120 hours with stirring in 100 mL of anhydromethylethyl ketone. After cooling of the reaction mixture, 300 mL of water was added for dissolution and neutralized. Thereafter, 300 mL of diethylether was added to the extract of the hydrophobic compounds. The ether layer was separated and evaporated. The residue, after evaporation, was distilled at vacuum condition. Thus, 3-hydroxy-4-dimethylamino toluene and 2-hydroxy-4-dimethylamino toluene, both of which are DMPT hydroxide compounds, were successfully prepared. No methoxy or benzene-methylated compounds were synthesized in this procedure.

In place of methyl iodide, the use of dimethylsulphonic acid was reported.[6] Following this method, methylation ability was so strong that not only an amino group, but also an aromatic hydroxy group (phenolic OH) was also methylated. Therefore, methyl iodide was used for selective methylation reactions.

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The linear gradient elution was carried out using a mixture of 10 mM ammonium acetate/acetonitrile combined with an HPLC column of Capcel Pak® C_{18} UG 120A (4.6 mm X 250 mm). For 40 min, a ratio of 10 mM ammonium acetate to acetonitrile was changed from 9 to one to one to 9. In order to increase sensitivity by MS detection, ammonium acetate was added to the eluent. Therefore, the addition of ammonium acetate was not for a common ion effect, but for increasing MS detection sensitivity. Flow rate was 1 mL/min, detection was by UV at 235 nm, and 10 μL of methanol extract of Yunifast® were injected into the HPLC of HP® 1050 from Hulett Packard Co. and the HPLC was connected to the MS of TSQ® 7000 from Finniganmat Co. in the atmosphere pressure chemical ionization (APCI) mode. The mother ion molecular weight and MS fragmentation for determining chemical structure was observed by the HPLC-MS-MS mode.

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Identification of Chemical Structure

Reproducible separation of hydrophilic compounds was attained from BA to MMA using linear gradient elution. Using the separation method, chemical structures of unidentified compounds eluted from BA to MMA was identified. Using HPLC-MS at APCI mode, only mother molecular weight results were obtained. Therefore, HPLC-MS-MS mode was essential to identify chemical structure from chemical fragmentation results in addition to mother molecular weight information. Inferiority of HPLC-MS at APCI mode was such that hydrophobic compounds were not sufficiently detected compared with other combination modes. The newly identified compounds in this case were found to be DMPT derivatives and, as they were aromatic amine hydrophilic compounds, thus they were successfully detected and the unidentified chemical structure was successfully identified in this experiment.

Determination of Unidentified Compounds in Saliva

Each three sheets of 3X3X0.1 cm of Yunifast® were immersed in 10 mL of saliva and the amount eluted to saliva was determined by HPLC combined with SPE. Pretreatment of newly identified DMPT derivatives (DMPT, epoxide DMPT, hydroxy DMPT) was carried out using the SPE C_{18} column in an identical manner to that of the DMPT pretreatment.

SPE Procedure of MMA, DMPT and DMPT Derivatives in Saliva

In saliva, BPO did not exist as it is and immediately transformed to BA, therefore, BPO in saliva was determined as BA in the following section.

There has been no reports on SPE with a satisfactory recovery of MMA, DMPT, BA (from BPO), and hydroxy DMPT in saliva. Epoxy DMPT was also immediately transformed to hydroxy DMPT in saliva in a few seconds. The SPE column used was Bond Elut® C_{18} with a void volume and resin weight of 120 μL and 100 mg, respectively.

SPE treatment of MMA, DMPT, and hydroxy DMPT was as follows: the C_{18} column was conditioned with 2 mL of acetonitrile and 2 mL of 50 mM phosphate buffer at pH 7.5. Thereafter, one mL of saliva was applied to the conditioned column, vacuumed, rinsed with 0.5 mL of 50 mM phosphate buffer at pH 7.5, and eluted with one mL of an alkalized acetonitrile with 50 mM phosphate buffer at pH 8. The drain was trapped and 20 μL were applied to HPLC. Conditioning, rinsing, and elution were carried out by a vacuum system.[2]

In the case of only MMA analysis, no alkalized eluent, such as 50 mM phosphate buffer at pH 8, was required due to neutral compound. As other compounds were basic, the simultaneous analysis of neutral and basic compounds of alkalized eluent was required.

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SPE Procedure of BA in Saliva

As mentioned above, BPO was stable in methanol, but when contacting with saliva or DMPT, BPO was immediately transformed to BA. Therefore, even though BA was not originally utilized in PMMA fabrication, BA existed as a sort of artifact from BPO during PMMA fabrication and during contact with body fluids such as blood or saliva.

SPE of BA in saliva was as follows: Depression of ionization of BA was essential to retain BA in a reverse-phase column. Thus, an acetic acid aqueous solution at pH 3 was added to the sample solution at a volume ration of 1/1 and mixed well prior to SPE application and carried out as follows: Bond Elut® C_{18} column was conditioned with 2 mL of acetonitrile and 2 mL of acetic acid aqueous solution at pH 3. One mL of saliva was applied to the conditioned column. Saliva was two-fold diluted with an acetic acid aqueous solution at pH 3 prior to the conditioned column application.

Thereafter, they were vacuumed, rinsed with 0.5 mL of acetic acid aqueous solution at pH 3 and eluted with one mL of acetonitrile acidified with acetic acid at pH 2.5. The drain was trapped and 20 μL were applied to HPLC. Conditioning, rinsing, and elution were carried out by a vacuum system.[2]

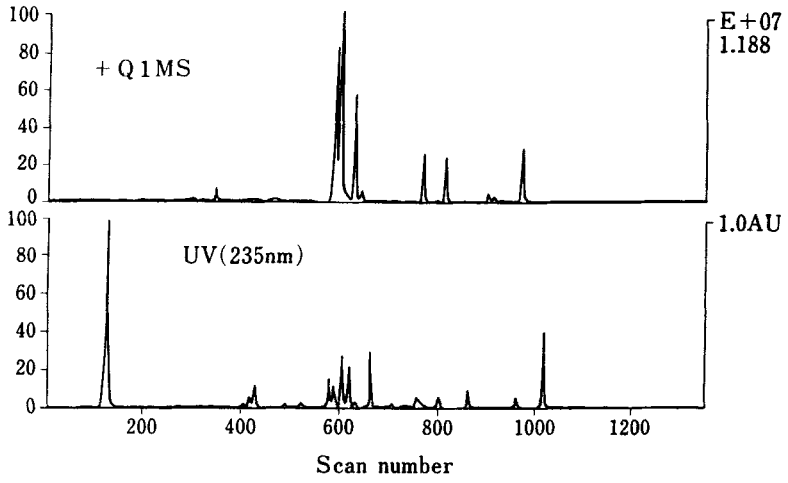

Figure 1. HPLC-MS MH^+ chromatogram BA: scan number of 130. Compounds of g): scan numbers of around 200 and 300, compounds of f): scan numbers of 340 and 460, compounds of e): scan numbers of around 520 and around 630 and compounds of a): scan numbers of around 580 and 590. Compound of b): scan number of 620. DMPT: scan number of around 810. MMA: scan number of around 610. 2,3-epoxy DMPT: scan number of around 340. One scan number corresponds to retention time of 2 seconds.

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RESULTS AND DISCUSSION

Figure 1 shows MS MH^+ (one protonated mother ion) chromatogram (upper) and gradient HPLC chromatogram (lower) of methanol extract detected by UV (235 nm). Background baseline slope was treated with a computer to attain flat baseline.

Figure 2 shows the chemical structure of identified compounds. Not all peaks in Figure 1 were identified.

Concerning the SPE procedure; if compounds have carboxy group, they were treated in an identical manner to BA treatment. Otherwise, an identical SPE proceure to MMA, DMPT, and BPO was used.

Neutral compounds of MMA and BPO were not affected by the pH of eluent, so the MMA and BPO elution procedure was different from the DMPT, basic compound, procedure. In the simultaneous analysis of basic and neutral compounds, identical procedures to DMPT analysis were used.

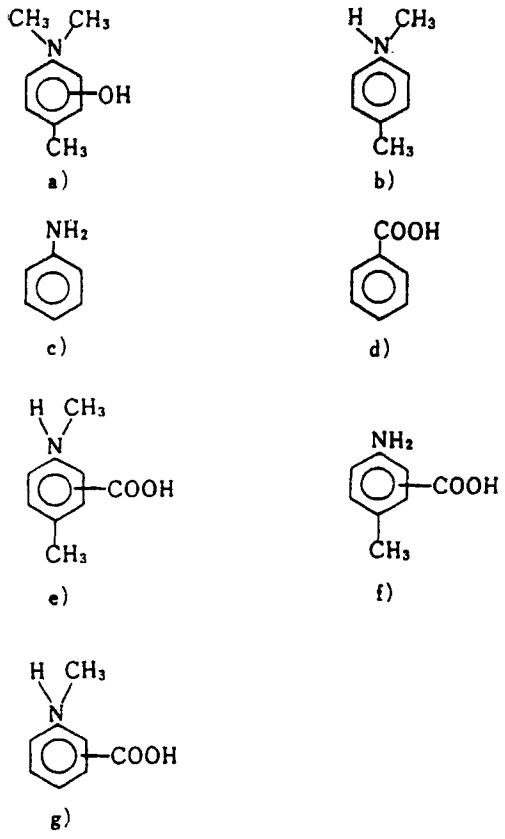

Figure 2. Chemical structure of compounds of a) to g) Compounds a): 2-hydroxy 4-dimethylamino toluene and 3-hydroxy 4-dimethylamino toluene, b): N-methyl *p*-toluidine, c): aniline, d): BA, e): 3-carboxy 4-N-methyl amino toluene and 2-carboxy 4-N-methyl amino toluene, f): 2-carboxy 4-amino toluene (3-amino 6-methylbenzoic acid) and 3-carboxy 4-amino toluene (2-amino 5-methylbenzoic acid), g):*o* and *p*-N-methyl amino benzoic acids.

The simultaneous analysis of basic, neutral, and acidic compounds was not successfully attained due to ion suppression at a lower pH eluent, which was opposite to the basic compound analysis which was required for acidic compound analysis.

When both carboxyl and amine groups exist in the chemical structure, as is the case of the newly identified compound, further study for appropriate SPE procedure will be required.

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Newly Identified Compounds

The newly identified compounds of hydroxy DMPT were recognized to elute from BA to MMA elution. BA elution was confirmed by MH^- (mother ion minus one proton). On the contrary, MMA and BPO, which were neutral compounds, were not detected by MS (MH^+ and MH^-) due to less vaporization characteristic. The elution of newly found compounds of hydroxy DMPT was confirmed by MS (MH^+), HPLC with UV detection and by the coincidence of elution time with synthesized compounds.

Unidentified hydrophilic compounds were determined by their chemical structure as hydroxylated derivatives of DMPT, 2,3-epoxy DMPT, and carboxylated DMPT derivatives from their molecular weight and MS fragmentation pattern, as well as, the coincidence of elution time of standard compounds. The molecular weight of 2 or 3 hydroxyl DMPTs and 2,3-epoxy DMPT were 151, 151, and 149 and their retention time was 6.7 min, 10 min, and 11.5 min, respectively. The retention time of BA, MMA, and DMPT was 4.3 min, 20.3 min, and 27 min, respectively.

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The elution of N-methyl-*p*-toluidine, which was not reported so far, was also confirmed to be eluted just after MMA elution. The retention time of this compound was 20.7 min and that of MMA was 20.3 min. The elution of this compound was confirmed from its fragmentation by MS. These compounds were confirmed from methanol extract of Yunifast® as well as the mixed solution of standard DMPT and BPO.

This possibility indicates that unidentified compounds may be produced from the reaction of DMPT and BPO during PMMA fabrication. This compound was also treated in the identical SPE manner as that of the DMPT treatment of SPE.

The 2,3-epoxy DMPT was stable in methanol solution, but when contacted with saliva, the epoxy compound changed immediately to 2 or 3 hydroxy DMPT. It was recognized that BPO was immediately converted to BA when BPO was contacted with saliva. As these compounds were highly reactive, it was speculated that they were highly toxic.

The degree of toxicity was not always parallel to the eluted amount. For example, serum extraction of MMA, DMPT, and BA from Yunifast® was 32.04 μg/g, 66.44 μg/g, and 2.3 μg/g, however the cytotoxicity data of IC50 (μg/mL) of MMA, DMPT, BA, and BPO using Balb 3T3 cell was 4400, 1500, 28.7, and 22. It is understandable that the greater elution with higher cytotoxicity may indicate a serious risk to human health.

It is a problem that the epoxide compound is thought to indicate the greatest toxicity, but as this compound will be transformed into a hydroxy compound immediately when contacting with saliva or blood, the cytotoxicity test for an epoxide compound was not successfully carried out.

The hydroxy DMPT compounds found in saliva were those originally existing in saliva, plus transformed compounds from 2,3-epoxy DMPT, but the differentiation of the origins of these compounds in saliva was extremely difficult; the attempt to seek differentiation was not fruitful. The eluted amount of 2- and 3-hydroxy DMPT into saliva, successively during three days was 10.7 μg/g and 15.8 μg/g (n=3), respectively. The amount of epoxy DMPT in saliva was not attained due to transformation immiediately to hydroxy DMPT. The elution of three successive days will be minimum due to putrefaction of saliva for further period immersion. Thus, the real elution amount to the treated patients will be much greater due to a much longer period of contact with dental material with saliva or blood through teeth during patient's life. The BPO in saliva was determined as BA at 3.5 μg/g.

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SPE Procedure of MMA, DMPT, and DMPT Derivatives

The basic compound of DMPT bound to the reversed-phase column was normally speculated to elute more favorably after DMPT was acidified to positively charge DMPT. Based on this speculation, the author initially carried out an elution experiment using an acidified methanol. However, the results were opposite to the speculated result, indicating the recovery rate was lower than alkalized methanol. The alkalized methanol elution indicated 100% recovery of DMPT from the C_{18} SPE column. The precise reason why the alkalized methanol was superior to the acidified methanol was speculated to be as follows: The alkalization will depress DMPT charge, but simultaneously stimulate silanol dissociation and acidification will be opposite. From these results, of which the amine charge in DMPT or the silanol charge in the column support may play a more essential role in elution characteristics. Binding of the DMPT to silanol was not significant from the experimental result using a silica column because of poor retention result. Acidified DMPT promotes charging of the DMPT amine, but this does not result in favorable recovery. As a result, both indicate amine and silanol in support will not be essential factors to be considered.

Finally, the author considers that the satisfactory result obtained with alkalized methanol may be due to a common ion effect. If so, the elution mechanism may be simpler than expected; however, the initially speculated procedure, which is in direct opposition to the common ion effect, has been often reported as the recommended SPE eluent. Additional speculation is that

the positively charged DMPT may not be sufficiently dissolved in hydrophobic eluent. If this is correct, the reason for the favorable result attained with alkalized methanol can be clarified. Thus, the undissociated DMPT will be more favorably dissolved in a hydrophobic eluent.

The author considers two major reasons for the favorable result using alkalized eluent for DMPT recovery from the C_{18} SPE column. One is the favorable dissolution into the organic solvent (methanol) eluent and the other is the common ion effect. The above speculation may be correct, but it was opposite to the conventionally reported result. The conventionally reported eluent seemed to have sound scientific rationale before carrying out the experiment. If the experimental result has significant reproducibility, which was quite essential to chemists, as reproducible data indicates a scientific truth.

The hydroxy DMPT has a phenolic OH, but its acidity is weak; therefore, this functional group did not affect the elution very much. The SPE procedure for epoxy DMPT was identical to that of original DMPT.

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SPE Procedure of BA

Concerning the SPE eluent of BA, acetonitrile, alkalized acetonitrile containing 50 mM sodium hydroxide or acidified acetonitrile adjusted to pH 2.5 with acetic acid were compared for elution. Acetonitrile alone showed an insufficient recovery (80%). Alkalized acetonitrile and acidified acetonitrile indicated 85% or 100% recovery, respectively; therefore, acidified acetonitrile was superior to alkalized acetonitrile. This result may be due to the favorable dissolution of the SPE eluent and common ion effect being identical to the SPE eluent of DMPT. Additional speculation was that an acidified solution was used during conditioning, so alkalinity may be suppressed due to acidified circumstance.

In the SPE of MMA and DMPT, 50 mM phosphate buffer at pH 7.5 was used for column conditioning. The use of water, or more than 50 mM phosphate buffer, resulted in lower recovery. One reason is that there was insufficient depression of DMPT ionization by water alone. The other reason is that excessive buffer ions at more than 50 mM will interfere with DMPT retention on the column.

In DMPT elution, alkalized acetonitrile (pH 8 phosphate buffer) was more effective than acetonitrile or acidified acetonitrile. These are favorable dissolutions to the eluent and common ion effect. Additional speculation was that an alkalized solution was used during conditioning, so acidity may be suppressed due to alkalized circumstance.

Acetonitrile alone also produced satisfactory recovery for MMA and BPO (neutral compound), but not for DMPT (strong basic compound). The reason for the favorable recovery for DMPT was identical to that of SPE eluention of BA. Since MMA and BPO are neutral compounds, they will not be affected by eluent pH.

As was previously mentioned, in SPE eluent for basic compounds of DMPT and DMPT derivatives, it was thought elution would be more favorable if DMPT was treated with an acidic solvent by positively charging the retained DMPT for easy removal from the solid resin. However, the experimental result was opposite to the expected result; the recovery rate with acidified methanol was lower than with alkalized methanol.

Favorable results have been produced, both when acidic acetonitrile was used for elution of acidic compound of BA and when alkalized acetonitrile was used for elution of basic compound of DMPT. These results were different from the already reported results for SPE eluent as recommended eluent; however, it is important that these experimental data are reproducible. Thus, it has a sound scientific rationale. Experimental results indicate any scientific truth, and the researcher carrying out the experiment should try to attain scientific truth.

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Liquid-Liquid Extraction Vs. SPE of MMA, DMPT, BPO and BA in Blood

Liquid-liquid extraction was carried out by adding an identical volume of acetonitrile to serum for deproteinization and extraction of MMA and DMPT. The MMA peak showed an insufficient separation from serum admixtures in HPLC and insufficient recoveries of MMA and DMPT (84% and 62% for MMA and DMPT, respectively, n=3).

Liquid-liquid extraction was a conventional pretreatment method for recovery, isolation, extraction, and purification of the compound of interest in complicated matrix such as body fluids. The inferior points of this method were copious consumption of organic solvent for extraction, requiring further condensation, which may result in loss of recovery during evaporation condensation, time consuming due to repeated extraction, or artifact formation of interest compound with extraction solvent.[7-12]

During a vacuum evaporation/condensation process, compounds of interest were often vaporized without being successfully trapped and may have causes a reduction of recovery rate and thermal decomposition.[7-12] Liquid-liquid extraction required a greater amount of consumption of organic solvents, which was hazardous to chemists handling them.

The recovery rate of a single treatment of liquid-liquid extraction was less than that of SPE because a single treatment of liquid-liquid extraction was almost identical to the SPE column with one theoretical plate. SPE, in general, did not require condensation and could be condensed using a less amount of eluent than applied sample volume.

In artifact formation, when ethyl acetate was used as an extraction solvent, compounds with hydroxyl or an amino group was acetylated, which caused a reduction of recovery rate. Artifacts of acetylated compounds are generally more mutagenic and toxic; thus this may lead to a misunderstanding by the researcher so that he/she may consider to extract strongly toxic compounds and, of course, this will cause a lower recovery rate. Another example of artifact formation was observed when an amine compound was extracted with methanol. Formaldehyde from methanol produced methylol and cross-linked both amine with methylene linkage. As a result of this oligomer, compounds were produced with methanol extraction. This reaction was named the Mannich reaction.

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This is the major reason the author always insists on the necessity of reproducible data with a satisfactory recovery rate; otherwise, the experimental data cannot be evaluated accurately due to insufficient results in attaining 100% recovery. Is this from an inappropriate solvent for recovery or artifact formation during extraction? No one can distinguish the difference and understand the truth. The researcher must clarify the truth for poor recovery of the experiments and try to find a more appropriate method to attain satisfactory recovery. Chemists should keep well in mind possible artifact formation during solvent extraction by reacting solvent with the compound of interest; otherwise, he/she may misunderstand the extraction of the stronger toxic compounds in his/her experiment. In that regard, almost 100% recovery rate with satisfactory reproducibility is most appropriate; otherwise, a lower recovery has a possibility of artifact formation during extraction. In order to avoid artifact formation, one approach is to select an appropriate extraction solvent. The other is to use SPE in place of liquid-liquid extraction.

CONCLUSIONS

Artifact formation during solvent extraction, including SPE, is problematic, but this phenomena was often overlooked. In order to avoid artifact formation to improve recovery rate, appropriate selection of the extraction solvent, which will be inert to the compound of interest, will be essential. Conventional liquid-liquid extraction compares poorly with SPE, therefore SPE pretreatment should be seriously considered because SPE was superior to liquid-liquid extraction in terms of less possibility of artifact formation, less consumption of solvents, less experimental time, greater recovery rate, fewer necessity of condensation, and so on.

As newly found toxic artifacts were produced during PMMA fabrication, they were successfully identified using HPLC-MS-MS at APCI mode. These were derivatives of starting compounds, mostly DMPT and BPO. During reaction of DMPT and BPO, most of newly identified compounds were produced. Some compounds were further converted to other compounds when contacting with saliva. As these compounds were toxic, appropriate SPE procedures, especially for compounds with both aromatic amine and carboxyl functional groups, will be further required. In place of a reverse phase column, the ion exchange column may be one candidate to examine.

Eluents of reverse phase columns for basic or acidic compounds were different from the conventionally reported results for SPE elution. Experimental results indicated the opposite result from the conventional speculation. It is more important to recognize that the experimental result has reproducibility. Reproducible results are essential as this indicates truth in science. Sound explanations for the experimental result with any scientific rationale was mostly due to common ion effect and favorable dissolution to the eluent.

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