COMPARISON OF LIPID EXTRACTION FROM THE SERUM BY THREE DIFFERENT METHODS

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Introduction

Many studies concerning adverse health effects of polychlorinated dibenzo-*p*-dioxins (PCDDs), polychlorinated dibenzofurans (PCDFs), dioxin-like polychlorinated biphenyls (DL-PCBs) and PCBs so far have been reported in particular on growth retardation of fetus and infants [1, 2], birth weight [3~7], neuro-developmental dysfunction [1, 2, 8~10], thyroid deficiency[1, 2, 11, 12], immune deficiency[1, 2, 13~16], reproduction and/or fertility [1, 2], diabetes [17~19]and cancer [1, 2]. In these studies, however, in order to evaluate their toxic effects, their concentrations on lipid weight basis have been employed, except for one study [12] and we have pointed out that this type of concentration is not good for their toxic and/or risk evaluations, due to the difficulties of reproducible and quantitative lipid extractions from biological samples such as the blood and breast milk [20~22] and also, of course, from other human tissues/organs. This is very important because in order to investigate health effects and risks of toxic compounds for humans, we usually use this kind of concentration, as mentioned above. Therefore, we are now inspecting the quantities and qualities of lipid extraction from the blood and serum by using several extraction methods.

In this study, we examined the qualitative and quantitative lipid extraction by three basic methods, namely, solvent systems of ethanol/n-hexane, chloroform/methanol and diethyl ether/petroleum ether, which were basically the same as the methods of Schecter and Ryan [23], Patterson et al. [24], and Nakagawa *et al.* [25], respectively.

Materials and Methods

Pooled serum which was free from syhlilis, hepatitis B and AIDS was made by CRC Inc., Fukuoka, Japan and freezed at -20^{o}C until employed. Three basic extraction methods of lipid from the serum (5ml) samples are simply summarized as follows:

1) Solvent system of ethanol/n-hexane (E.H.): 3ml saturated ammonium sulfate and 12ml ethanol/ n-hexane (1:3 v/v) were added to each sample and shaked for 30 min. The upper hexane phase was transferred to a separate container and the lower aqueous-ethanol phase was extracted anew with 10ml n-hexane for 30 min and this was repeated again. The combined hexane phase was concentrated near to 5ml, washed with 10ml water three times and treated with anhydrous sodium sulfate. The extract was evaporated to dryness and the amount of lipid was determined gravimetrically.

2) Solvent system of chloroform/methanol (C.M.): 15ml chloroform/methanol (1:2 v/v) was added to each sample and shaked for 30 min and then this mixture was extracted with 17.5ml chloroform/methanol/water (2:3:2 v/v) for 30 min. The upper water phase was transferred to a separate container and extracted with 12.5ml chloroform/methanol (2:3 v/v) for 30 min and this was repeated again with chloroform/methanol (4:1 v/v). The combined chloroform phase was extracted with 70ml n-hexane for 5 min and washed with 10ml water three times. The hexane extract was evaporated to dryness and the amount of lipid was determined gravimetrically

3) Solvent system of diethyl ether/petroleum ether (E.P): 0.7ml 10% potassium oxalate solution and 7ml ethanol were added to each sample and the mixture was treated with ultrasonic for 30 sec. and then shaked with 7ml diethyl

ether/petroleum ether (1:1 v/v) for 5 min. The upper ether phase was transferred to a separate container and the lower aqueous ethanol phase was extracted anew with 4ml diethyl ether/petroleum ether for 5 min and this was repeated again. The combined ether phase was washed with 7ml water three times and treated with anhydrous sodium sulfate. The extract was evaporated to dryness and the amount of lipid was determined gravimetrically.

The efficiency of lipid extraction was also examined by additional two modified methods in each of these three methods.

The first modification: in the first stage of lipid extraction, serum was continuously dropped into each solvent system, that is, 3ml saturated ammonium sulfate and 3ml ethanol in E.H., 10ml methanol in C.M. and 0.7ml 10% potassium oxalate and 7ml ethanol in E.P., which were being stirred with stirrer. After adding the remaining solvents in E.H. and C.M., that is, 9ml n-hexane and 5ml chloroform, respectively, the mixtures of the three solvent systems were slowly shaked for 30 min at 40°C. The remaining procedures were the same as those in the three basic extraction methods and this was expressed as the dropping- dispersion method of each basic method.

The second modification: before dropping into each solvent system, 0.5ml sodium dodecyl sulpate (SDS) was added to 4.5ml serum at the final concentration of 0.5%. The remaining procedures were the same as those in the three dropping-dispersion methods and these were expressed as SDS methods.

Estimates of total lipids of the serum were calculated by summation of the individual four lipid components of the serum by the following formulas [26].

$$TL = 1.677 (TC – FC) + FC + TG + PL$$

Where TL is total lipid, TC is total cholesterol, FC is free cholesterol, TG is triglyceride and PL is phospholipid, all expressed in unit of mg/dl. Concentrations of TC, FC, TG and PL of the serum were measured using standard enzymatic and automatic clinical procedures on a Hitachi 7600-300s for TC and TG and on a BM 9130 for FC and PL in CRC Inc., Fukuoka, Japan.

Concentrations of TL of the serum and extracted by the six methods were statistically examined by Student's T test.

Results and discussion

The concentrations of TC, FC, TG and PL in the pooled serum were 144, 30, 70 and 170mg/dl, respectively. Orders of these concentrations extracted by the three basic methods as compared to the respective concentrations of the pooled serum as 100 were as follows; TC : C.M. (98) > E.H. (89) > E.P. (83), FC: C.M. (100) ≥ E.H. (97) > E.P. (87), TG: C.M. (99) > E.H. (87) ≥ E.P. (83), PL: C.M. (56) ≥ E.H. (53) > E.P. (43). Therefore, as indicated in Fig. 1, estimated concentrations of TL calculated by the four components was the highest in C.M.. However, due to the low extraction rates of PL in the three methods, ranges in estimated concentrations of TL to that of the serum were 68 to 83%. Compared to the basic extraction methods, extraction efficiencies of lipid components were a little bit better in the dropping-dispersion methods, except for those of TC and TG in E.P. In the SDS methods, extraction efficiencies of PL were worse than those of the basic methods. In particular, only 0.6% of PL could be extracted from the serum in the SDS method of E.H.. Therefore, as shown in Fig. 2, estimated concentration of TL in the SDS method of E.H. was almost the same as that in the SDS method of E.P..

At present, among the six extraction methods, the dropping-dispersion method in C.M. is considered the best, in which estimated concentration of TL was 85% of that of the serum. In conclusion, in order to establish the reproducible and quantitative method for lipid extraction, the most difficult problem is the extraction of PL, as we have reported before [22, 27, 28]. Therefore, we have to develop the best solvent system for this, as soon as possible.

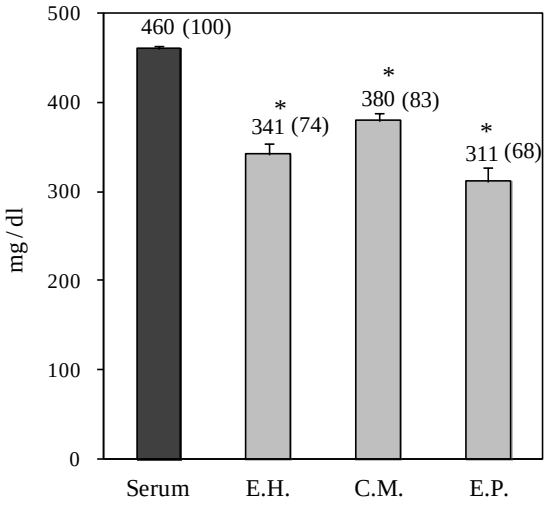

Fig. 1. Comparison in estimated concentrations of total lipid calculated by four components in the basic extraction methods.
Figures in parentheses; % to the serum (100)
*: $p < 1 \times 10^{-8}$

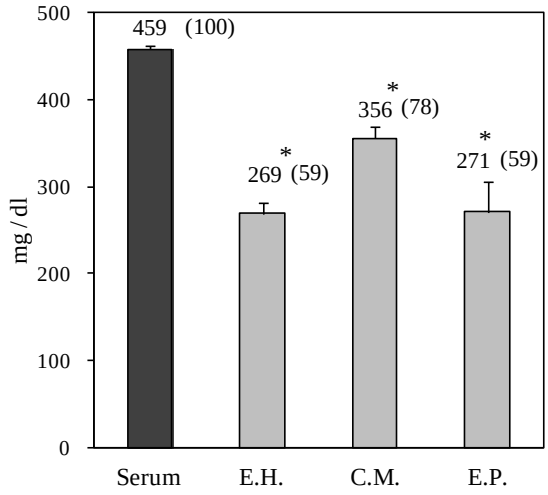

Fig. 2. Comparison in estimated concentrations of total lipid calculated by four components in the SDS methods.
Figures in parentheses; % to the serum (100
*: $p < 0.01$

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