ASIAN JOURNAL OF PHARMACEUTICAL AND CLINICAL RESEARCH

INNOVARE ACADEMIC SCIENCES
Knowledge to Innovation

Vol 11, Issue 3, 2018

Online - 2455-3891
Print - 0974-2441

Research Article

DETERMINATION OF DOCOSAHEXAENOIC ACID IN INFANT FORMULAS WITH GAS CHROMATOGRAPHY

HARMITA HARMITA*, UMAR MANSUR, STEPHANIE STEPHANIE

Faculty of Pharmacy-University of Indonesia, Depok, Indonesia. Email: igakadeharmita@gmail.com

Received: 08 November 2017, Revised and Accepted: 07 December 2017

ABSTRACT

Objective: Docosahexaenoic acid (DHA) is important for the development of infant's nervous and visual system because it is a major fatty acid in brain and retina phospholipids. However, the benefit of adding DHA in infant formulas is still controversial. The over intake of DHA should be considered because of its side effect. The aim of this study was to get a valid analysis method of DHA using gas chromatography (GC) to determine the concentration of DHA in infant formula.

Method: The milk fat was extracted in chloroform-methanol (1:2), continued with methylated in methanol-toluene (4:1) with acetyl chloride, and finally, injected to GC.

Result: The GC conditions were as follows: Injector temperature was 230°C, detector temperature was 250°C, oven temperature was programmed to increase from 130°C to 230°C by 2°C/min and held for 20 min, helium flow rate was 2.00 ml/min, and split ratio was 1:3. This method had passed the precision and recovery evaluation. The result of DHA determination in five infant formula samples was 27.49±0.62 mg/100 g, 31.14±0.43 mg/100 g, 11.83±0.38 mg/100 g, 19.34±0.58 mg/100 g, and 45.87±0.42 mg/100 g.

Conclusion: The method was valid and successfully applied to determine of DHA in infant formula.

Keywords: Docosahexaenoic acid, Gas chromatography, Infant formula.

 DOI: http://dx.doi.org/10.22159/ajpcr.2018.v11i3.23530

INTRODUCTION

Quality of human being determined by the early growth and development. The right intake of nutrients is essential for the optimal development of genetic potency. The nutrients must be given correctly both the quality and the quantity [1,2]. Mistakes in feeding will affect the quality of human being in the future. It is mainly related to the growth and development of vital organs, especially the brain that mostly grows fast during the last trimester of pregnancy and 1st months of life. This growing brain needs perfect nutrients intake [3].

Docosahexaenoic acid (DHA) is a major fatty acid in brain and retina phospholipids [4]. It has important functional membrane and cellular properties of neural tissue [5]. Human milk contains DHA, whereas infant formula, which made from cow milk, has no DHA. To fulfil the necessity of DHA as essential nutrient, DHA is added to infant formulas [3], but the benefit of DHA in infant formulas is still controversial [1].

Over intake of DHA can inhibit the formation of arachidonic acid and also compete with arachidonic acid for the cyclooxygenase, and thus reduce the formation of prostaglandin Histamine 2 and Histamine 3, thromboxane, and leukotriene that can inhibit inflammatory and immune response, which cause longer time of bleeding and decrease renin that has important role in renal regulatory [5].

The analysis method of DHA needed to avoid improper DHA concentration in infant formulas. The analysis method of DHA is not simple. First, the milk fat has to be separated from other components such as carbohydrate and protein. Then, before injected to GC, the fat has to be converted to methyl ester [6,7]. The extraction and esterification steps must be carried out carefully to avoid the losses of DHA, because of these complicated steps, the analysis method of DHA needs to be studied.

METHODS

Equipments

Gas chromatography (GC) (Shimadzu GC-17A), VB-wax capillary column (60 m×0.32 mm×0.25 μm) equipped with a flame-ionized detector, helium as carrier gas, Class GC solution data processor, and microsyringe 10 μl (SGE), oven, centrifuge (Kubota 6800 and 5100), analytic, borosilicate glass tubes with feflon-lined screw-caps (100 mm×13 mm), centrifugation tubes, vortex, micropipette (Socorex), and other glass-wares for quantitative analysis were commonly used.

Materials

DHA (Sigma, CAS no. 6217545), DHA oil (Tama Biochemical Co. Ltd. Lot 611151), methanol p.a. (Merck), chloroform p.a. (Merck), toluene p.a. (Merck), hexane p.a. (Merck), acetyl chloride for synthesis (Merck), sodium chloride p.a. (Merck), and potassium carbonate p.a. (Merck) were used.

Determination of GC analysis condition

About 25 mg of DHA standard was precisely weighed, then diluted with hexane until 10.0 ml, 300 μl of this 2500 μg/ml DHA standard solution were placed to teflon-lined caps reaction tube, dried under nitrogen stream, and was submitted to the esterification procedure described below. 1.0 μl of the aliquot of the upper toluene phase was injected into the chromatograph. The experiments to determine the GC analysis were done in isothermal condition and by temperature programmed. Elusion with isothermal condition was done by holding the temperature at 200°C with helium flow rate 1.35 ml/min. Elusion with temperature programmed was done by variation of the starting temperature at 120, 130, and 140°C and also a variation of flow rate at 1.35, 1.80, and

2.00 ml/min. The column temperature was increased 2°C/min until 230°C then was held at 230°C for 20 min. The split ratio was 1:3. The injector port temperature was 230°C and the detector was 250°C. The condition that had the highest value of theoretical plates and the lowest value of HETP were chosen on the next steps of this research [8].

Determination of DHA concentration in DHA oil

50 µl, 100 µl, 200 µl, 300 µl, 400 µl, and 500 µl of the 2500 µg/ml DHA standard solution were placed to Teflon-lined caps reaction tubes, dried under nitrogen stream, and were submitted to the esterification procedure described below. 1.0 µl of the aliquot of the upper toluene phase was injected into the chromatograph. Each area of DHA chromatograph was used to make calibration curve, then calculated the regression equation.

About 25 mg of DHA oil was precisely weighed, then diluted with hexane until 25.0 ml, 300 µl of this 10000 µg/ml DHA oil solution were placed to Teflon-lined caps reaction tube, dried under nitrogen stream, and was submitted to the esterification procedure described below. 1.0 µl of the aliquot of the upper toluene phase was injected to the chromatograph with chosen analysis condition. The concentration of DHA was calculated by inserting the area of DHA chromatograph to the calibration curve equation. This experiment was repeated twice.

Linearity test, calibration curve, and calculation of limit of detection (LOD) and limit of quantification (LOQ)

50 µl, 100 µl, 200 µl, 300 µl, 400 µl, 500 µl, and 600 µl of the 10000 µg/ml DHA oil solution were placed to Teflon-lined caps reaction tubes, dried under nitrogen stream, and were submitted to the esterification procedure described below. 1.0 µl of the aliquot of the upper toluene phase was injected into the chromatograph. Each area of DHA chromatograph was used to make calibration curve and then calculated the regression equation. Linearity was showed by the value of the coefficient of correlation between DHA concentration and area of DHA chromatogram.

Precision test

100 µl, 200 µl, and 300 µl of 10000 µg/ml DHA oil solution were put into Teflon-lined caps reaction tubes, dried under nitrogen stream, and were submitted to the esterification procedure described below. 1.0 µl of the aliquot of the upper toluene phase was injected to the chromatograph. Each concentration was repeated 5 times. Precision calculated as the coefficient of variation.

Recovery test

About 90 mg of DHA oil was precisely weighed, then diluted with hexane until 10.0 ml. 2 g of milk that contain no DHA were put into each 50 ml centrifugation tube, and 200 µl, 250 µl, and 300 µl of 90000 µg/ml DHA oil solution were added into each tube. Then, the mixtures were treated for the extraction and esterification procedure described below. 1.0 µl of the aliquot of the upper toluene phase was injected into the chromatograph. Each concentration was repeated twice. Recovery calculated by comparing the obtained concentration to the actual concentration of DHA added.

Extraction of milk fat

About 2 g of milk sample was put into centrifugation tube. 15 ml chloroform-methanol (1:2) was added into it [9,10], and the tube was shaken well for about 15 min in orbital shaker. Then, 5 ml of chloroform was added and vortex well. Next, 5 ml of 9% sodium chloride solution was added and vortexed well. After that, the tube was centrifuged at 3000 rpm for 5 min. It would form 3 layers. The upper phase and middle phase were discarded slowly and carefully. The bottom phase was washed with 10 ml of methanol-saline solution (9:10) and vortexed well. The bottom phase was collected into Erlenmeyer, and the chloroform was vaporized by heating on the water bath. The fat extract was determined gravimetrically.

RESULTS AND DISCUSSION

GC analysis condition

The obtain GC analysis conditions were as follows: Injector temperature was 230°C, detector temperature was 250°C, oven temperature was programmed to increase from 130°C to 230°C by 2°C/min and held for 20 min, helium flow rate was 2.00 ml/min, and split ratio was 1:3. Among the other condition, it had the highest value of theoretical plates or the lowest value of HETP [11]. The results clearly showed in Table 1 and Fig. 1.

Determination of DHA in DHA oil

From triple experiments, the average DHA concentration in DHA oil was 22.76%. The measurement of DHA in DHA oil was plotted to the linear regression equation of the calibration curve of DHA standard. The equation of calibration curve of DHA standard was y = −3349.6516 + 66.8654x, with a value of the coefficient of correlation was 0.9999.

Esterification of fat

The fat extract was put into Teflon-lined caps reaction tube and dissolved in 0.40 ml of toluene and 1.6 ml of methanol, then it was shaken well. 0.2 ml of acetyl chloride was added slowly over 1 min while the tube was shaken slowly. Tube was closed tightly and methanolysis was conducted at 100°C for 1 h. After tube had been cooled in water, 5 ml of 6% K_2CO_3 solution was added slowly to stop the reaction and neutralize the mixture. The tubes were shaken and centrifuged, and an aliquot of the toluene upper phase was injected into the chromatograph. The correlation was 0.9993. The result clearly is presented in Table 2 and 3.

Linearity test, calibration curve, and calculation of LOD and LOQ

The linear regression equation of calibration curve was y=−356.1393+67.1206x, with a value of the coefficient of correlation

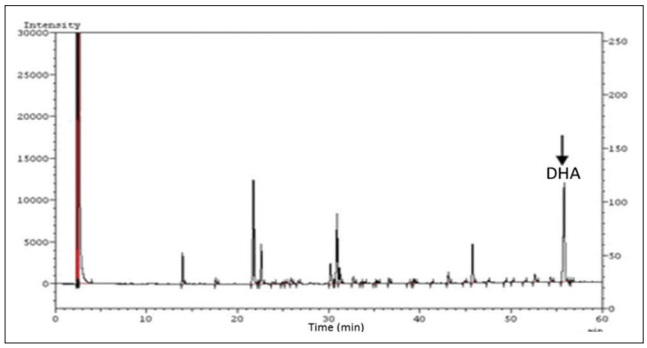

Fig. 1: Chromatogram of docosahexaenoic acid oil

Table 1: Determination of gas chromatography analysis condition

Condition	Starting (°C)	Flow rate (mL/min)	Time retention (min)	Theoretical plates	HETP
Programmed	120	1.35	68.200	605654.984	0.00991
	120	1.80	63.468	686485.845	0.00874
	120	2.00	62.116	671114.224	0.00894
	130	1.35	62.686	638605.624	0.00934
Isothermal	140	1.35	56.731	587010.084	0.01022
	140	1.80	52.439	523527.926	0.01146
	140	2.00	51.029	501615.256	0.01196
	200	1.35	68.280	-	-

was 0.9999. With the obtained value of the coefficient of correlation, the calibration curve of DHA concluded linear. LOD was 74.33 µg/g and LOQ was 247.77 µg/g. These values were still below the smallest concentration in the calibration curve.

Precision test

In this research, precision test was conducted at low, medium, and high concentration of calibration curve. The concentrations were 689.95 µg/g, 2051.45 µg/g, and 4066.25 µg/g. The precision of esterification and chromatography analysis of DHA was good with the coefficient of variations of each concentration, respectively, was 1.73%, 1.46%, and 1.85%. Since the coefficient of variations was below 2%, this method concluded having a precise result. The result clearly is shown in Table 4.

Table 2: Result of DHA standard measurements for calibration curve

Concentration (µg/g)	Area (µV/s)
388.58	24495
776.85	50342
1552.50	99519
2326.94	146129
3100.19	203598
3872.23	259360

DHA: Docosahexaenoic acid

Table 3: Result of DHA determination in DHA oil

Area (µV/s)	Concentration (µg/ml)	DHA concentration (%)	Average concentration (%)
133947	2389.711	23.78	
133956	2389.868	23.78	22.76
133573	2383.201	23.72	

DHA: Docosahexaenoic acid

Recovery test

Recovery test was conducted using absolute method, which calculated as the percentage of recovery of DHA was added to the blank milk. The amount of DHA that added to the blank milk was 0.025% from total weight of milk. This evaluation had a satisfactory result with the percentage of recovery of DHA was 96.40%. The recovery requirement which has concentration below 0.1% is 95.0–105.0%. With this result, it concluded that the analysis method used was accurate enough. The result clearly showed in Table 5.

Result of DHA determination in some infant formulas

After determining five different samples of infant formulas, three samples had DHA concentration significantly higher than the concentration written in the packaging label. DHA concentrations in five samples, respectively, were 27.49±0.62 mg/100 g, 31.14±0.43 mg/100 g, 11.83±0.38 mg/100 g, 19.34±0.58 mg/100 g, and 45.87±0.42 mg/100 g.

Sample A had DHA 8.36% less than the concentration written in packaging label, sample B had 55.68% more DHA, sample C had 51.70% more DHA, sample D had 136.44% more DHA, and sample E had 5.44% more DHA than the concentration written in packaging label. The result clearly showed in Table 6.

CONCLUSION

The optimum GC analysis conditions were as follows: Injector temperature was 230°C, detector temperature was 250°C, oven

Table 4: Result of precision test

Concentration (µg/g)	Area (µV/s)	Measured Concentration (µg/g)	Mean measured Concentration (µg/g)	Standard of deviation	Coefficient of variation (%)
	49277	740,338			
	47014	706,118			
689.95	48238	724,627	721.164	12.4506	1.73
	47500	713,467			
	47603	715,010			
	48662	731,038			
	130731	1972,039			
	133947	2020,669			
2051.45	133956	2020,805	1975.261	28.7647	1.46
	131826	1988,597			
	129315	1950,627			
	133573	2015,014			
	271621	4102,496			
	273395	4129,322			
4066.25	282373	4265,082	4110.744	75.5626	1.84
	280094	4230,620			
	269217	4066,144			
	276658	4178,663			

Table 5: Result of recovery test

Added DHA (µg)	Result of DHA determinations		
	Measured (µg)	Recovery (%)	Mean Recovery (%)
	407.928	97.20	
419.694	402.315	95.86	96.27
	401.834	95.74	
	512.903	97.77	
524.618	499.957	95.30	96.05
	498.857	95.09	
	596.784	94.80	
629.542	616.623	97.95	96.88
	616.342	97.90	

DHA: Docosahexaenoic acid

Table 6: Result of determination in some infant formulas

Sample	Result of DHA determination		
	Concentration (mg/100 g)	Mean concentration (mg/100 g)	Concentration written in the packaging label (mg/100 g)
A	27.46 26.89 28.13	27.46	30.0
B	31.46 30.64 31.31	31.14	20.0
C	11.96 11.41 12.14	11.83	7.8
D	19.98 19.21 18.83	19.34	8.2
E	45.69 45.57 46.34	45.87	43.5

DHA: Docosahexaenoic acid

temperature was programmed to increase from 130°C to 230°C by 2°C/min and held for 20 min, helium flow rate was 2.00 ml/min, and split ratio was 1:3. The extraction, esterification, and chromatography method of DHA determination used in this research had passed the linearity, precision, and recovery evaluation so that this method can be applied to determine DHA in infant formula. DHA concentrations in five infant formula samples, respectively, were 27.49±0.62 mg/100 g, 31.14±0.43 mg/100 g, 11.83±0.38 mg/100 g, 19.34±0.58 mg/100 g, and 45.87±0.42 mg/100 g.

AUTHORS CONTRIBUTIONS

All the author have contributed equally.

CONFLICT OF INTERESTS

Declared none.

REFERENCES

1. Judarwanto W. Kontroversi Penambahan AA dan DHA pada Vitamin dan Makanan Bayi. Available from: http://www.eppa.multiply.com/journal/item/115. [Last cited on 2016 Sep 10].
2. Nikhade R, Deshpande SA. Formulation evaluation and validation of ophthalmic emulsion of docosahexaenoic acid. Int J Pharmacol Res 2014;4:67-70.
3. Hidajat B. Penambahan DHA dan AA pada Bayi: Peran dan Manfaatnya. Available from: http://www.pediatrik.com/ilmiah_popular/2006 0220-ozgay7-ilmiah_popular.doc. [Last cited on 2016 Sep 08].
4. Willatts P, Forsyth JS, DiModugno MK, Varma S, Colvin M. Effect of long-chain polyunsaturated fatty acids in infant formula on problem solving at 10 months of age. Lancet 1998;352:688-91.
5. WHO. Fats and Oil in Human Nutrition. Report of a Joint Expert Consultation. Rome: WHO; 1993. p. 19-26.
6. Skoog DA, Donald MW, James FH, Stanly RC. Fundamentals of Analytical Chemistry. 9th ed. Brookly (USA): Thomson Learning Inc; 2014.
7. Bligh EG, Dyer WJ. A rapid method for total lipid extraction and purification. Can J Biochem Physiol 1959;37:911-7.
8. Sahoo S, Jena S, Sahoo A, Ray A, Sudan I. GC-MS profile of *in vivo* and *in vitro* shoots of *cleome gynandra* L. Int J Pharm Pharm Sci 2017;9:21-6.
9. Lepage G, Roy CC. Direct transesterification of all classes of lipids in a one-step reaction. J Lipid Res 1986;27:114-20.
10. Or-Rashid MM, Odongo NE, Wright TC, McBride BW. Fatty acid profile of bovine milk naturally enhanced with docosahexaenoic acid. J Agric Food Chem 2009;57:1366-71.
11. Nongalleima K, Ajungla T, Chingakham BS. GC-MS based metabolic profiling of essential oil of *Citrus macroptera* montruz leaves and peel, assessment of *in vitro* antioxidant and anti-inflammatory activity. Int J Pharm Pharm Sci 2017;9:107-14.