QUANTITATIVE DETERMINATION OF FATTY ACIDS IN INFANT FORMULA BY GAS CHROMATOGRAPHY WITHOUT DERIVATIZATION

M. R. Oveisi[1], N. Sadeghi[1*], M. Hajimahmoodi[1], B. Jannat[2], A. Behfar[3] and H. Sobhani[1]

1) Department of Drug and Food Control, Faculty of Pharmacy, Tehran University of Medical Sciences, Tehran, Iran

2) Ministry of Health and Medical Education, Tehran, Iran

3) Department of Bromatology, Faculty of Pharmacy, Ahwaz University of Medical Sciences, Ahwaz, Iran

Abstract- Fatty acids play important roles in biological systems and the newborns fatty acids requirements are covered only by the milk. It is of particular interest to qualify the content of the fatty acids in the milk. This study was performed to determine the levels of some fatty acids in the infant formulas and also to describe a method without derivatization for the fatty acids analysis and applying it to the control of infant formulas. Free fatty acids were produced by adding isopropanol- KOH to milk fat extract and heating it to saponify and acidify by H_2SO_4. Free fatty acids were extracted and were quantified by capillary gas chromatography on a fused silica column (AT-1000) and flame ionization detector. The average experimental values of lauric, palmitic, stearic and linoleic fatty acids contents of twenty infant formulas were 6.47, 16.52, 2.11 and 14.56 g/100g, respectively. The obtained experimental values of lauric and linoleic fatty acids contents of twenty infant formulas were in good agreement with the values proposed by standards of codex alimentary.

INTRODUCTION

The fatty acids play important roles in the biological systems. They are the constituents of the lipids in the biological membranes which influence membrane properties such as the fluidity, integrity, permeability and the activities of the membrane bound enzymes (1). Food's fatty acids also serve as the source of energy for man. It's also important to intake enough fatty acids for newborn and children especially for brain growth.

* Corresponding Author:
N. Sadeghi, Department of Drug and Food Control, Faculty of Pharmacy, Tehran University of Medical Sciences, Tehran, Iran
Tel: +98 21 66959090-2029,
Fax: +98 21 66461178
E-mail: nsadeghi@sina.tums.ac.ir

As the only dietary intake of the newborn is the milk, it is of particular interest to qualify the intake of the fatty acids in the milk. Infant formulas are widely used as substitutes for human milk, although the latter is considered as the best food supply for young infants (2, 3). There are some reports on the analysis of fatty acids in infant formula with gas chromatography of their fatty acids methyl esters (FAMEs) (4-6). The gas chromatography analysis of FAMEs consists of several steps, including fatty acid saponification, esterification, injection, separation, identification and quantitation. Each of these steps has to be optimized in order to attain high accuracy and precision of analysis. Derivatization methods, which involve solvent extraction, purification, derivatization and gas liquid chromatography, are expensive, lengthy and cumbersome. Furthermore, dangerous reagents such as BF3 which is very toxic,

inflammable and sensitive to moisture are to be used. Underivatization method is more specific and permits measurements of free fatty acids with high accuracy (7). Determination of fatty acids without derivatization has special application in food control.

The aim of this study was to determine the levels of some fatty acids in the infant formulas and to describe a method for the analysis of fatty acids without derivatization and applying it to the control of the infant formulas.

MATERIAL AND METHODS

Reagents and chemicals

All reagents were of analytical-reagent grade and bidistilled water was used throughout.

Standard grades of lauric, palmitic, stearic and linoleic acids were purchased from Sigma (St. Louis, MO, USA). Potassium hydroxide, sulfuric acid and the organic solvents: hexane, butanol and isopropanol were obtained from Merck (Darmstadt, Germany).

Gas chromatography analysis

Gas chromatography analyses were performed on a model 1000 DPC gas chromatograph (DANI, Milano, Italy) equipped with a split/splitless capillary (1:40) injector and a flame ionization detector. Analytical separation was achieved on an AT-1000 capillary column (25 m × 0.32 mm i.d.) with 0.2 µm film thickness (Alltech, Illinois, USA). Nitrogen was used as carrier gas (with a constant flow rate of 1 ml/min).

The air, hydrogen and auxiliary gas (N_2) pressures for detector were kept at 1.1, 0.9 and 0.8 bar, respectively. Temperature setting was as follows: injector 250ºC, and detector 280ºC. The oven temperature was held at 42ºC for 0.2 min, programmed to 100ºC at 25ºC/min, and held for 6 min then programmed to 240ºC at 8ºC/min, and held for 15 min. The peak areas were used to calculate the fatty acid content. The fatty acids chosen for controlling the infant formula were lauric (12:0), palmitic (16:0), stearic (18:0) and linoleic (18:2) acids.

Standard preparation

Stock solutions of lauric, palmitic, stearic and linoleic acids (100 mg/ml) were prepared in mixture of hexane, butyl alcohol solution (100:1). Stock solution of internal standard was prepared by dissolving 0.1 g phenol in 10 ml mixture of hexane, butyl alcohol solution (100:1).

Suitable volume of stock solutions of fatty acids and 1 ml of internal standard solution were used for preparation of working standards (2.5, 5, 10, 15, 20 mg/ml).

Sample preparation

Milk emulsion was prepared by adding 50 g of milk powder to 500 ml of distilled water. The emulsion was broken by 20 ml of 65% nitric acid on the heat. This mixture was centrifuged at 10000 rpm for 10 minutes to separate solid phase that contains fat and proteins. Proteins were denatured by vigorously mixing the solid phase with 20 ml of ethyl alcohol and heating in boiling water bath tub for 10 minutes.

After adding 100 ml of petroleum ether and mixing well the mixture was centrifuged again at 10000 rpm for 10 minutes. Organic phase was separated and petroleum ether was evaporated without heating. The fat was analyzed immediately after extraction and the stock was kept at 4ºC in screw-cap vials.

A mass of 0.5 g well-mixed melted fat was transferred to a tube with lip and 5 ml of 50 mg/ml of KOH in isopropanol was added. The tube was placed 5 cm deep in a boiling water bath tub for 30 minutes to saponify the oil and to evaporate the isopropanol.

A volume of 0.35 ml of a mixture of concentrated H_2SO_4/distilled H_2O (2:1) was added drop wise to the soap while the tube was cooled in cold water. Lumps at the bottom of the tube were broken using a glass-stirring rod.

A yellow mixture containing fatty acids clinging to a viscous aqueous K_2SO_4 layer was obtained. A volume of 10 ml hexane: butanol (100:1) solution was added to the tube and mixed thoroughly with a glass rod. The organic solution was filtered using a 0.45 µm filter. A volume of 1 µl of the filtrate was analyzed by gas chromatography.

Table 1. Fatty acid contents of twenty infant formulas

	Lauric acid	Palmitic acid	Stearic acid	Linoleic acid
Value (g/100g total fat)*	6.47 ± 0.32	16.52 ± 0.12	2.11 ± 0.45	14.56 ± 0.24
Standards of I.S.I.R.I.	5-7	19-27	5-10	9-16

Abbreviation: ISIRI, Institute of Standard Industrial Research of Iran.
*Data are given as mean $\pm$ SD.

RESULTS

Fatty acid composition in infant formula

Figure 1 shows a chromatogram obtained using this method to analyze infant formula samples. The average obtained experimental values of fatty acid contents from twenty infant formulas are listed in Table 1.

Linearity and analytical range

The correlation between the peak areas ratios of fatty acid standards to the internal standard solution were evaluated over the range 0-20 mg/ml. The results were found to be linear and are shown in Table 2. The chromatograms of fatty acid standards are shown in Figure 1.

Precision

The repeatability of the method was calculated by using the measured data of a single day. In contrast internal reproducibility of the method was calculated by using the measured data of 5 successive days. Both values were expressed by relative standard deviation. The precision data are shown in Table 3.
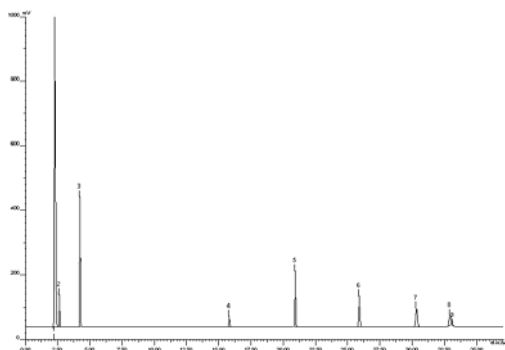
Accuracy

The accuracy of the method was verified by means of recovery assay (8). This was accomplished by an analyzing standard solution and spiked (enriched) samples. The average recovery obtained for lauric, palmitic, stearic and linoleic fatty acids were 91.5, 92.3, 93.5 and 92.8%, respectively.

Limit of detection

Three folds of standard deviation (SD) of blank hexane were used for evaluation of limit of detection (8). For fatty acids lauric, palmitic, stearic and linoleic it was 1.7, 2, 2 and 1.9 µg/ml, respectively.

Limit of determination

Ten folds of SD of blank hexane were used for calculation of limits of determination (8). In the same order that mentioned for fatty acids, it was 6, 7, 8 and 7 µg/ml, respectively.

Sensitivity

By calculating slopes of calibration curves (8) the sensitivity for the same order of the above fatty acids was 0.24, 0.17, 0.14 and 0.04 RAUC / (mg/ml), respectively.

Fig. 1. Chromatographic fatty acid profiles of the infant formula. 4, phenol; 5, Lauric acid; 6, Palmitic acid; 7, Stearic acid; 8, Linoleic acid.

Table 2. Linearity parameters of four fatty acids

Fatty acid	Calibration curve equation	Correlation coefficient (R^2)
Lauric acid	$Y = 0.2398 + 0.2218X$	0.9917
Palmitic acid	$Y = 0.1709 + 0.2110X$	0.9940
Stearic acid	$Y = 0.1391 + 0.2162X$	0.9950
Linoleic acid	$Y = 0.0354 + 0.1505X$	0.9952

Table 3. Precision (RSD %) of the method for four fatty acids*

Concentration	Repeatability (n = 10)				Internal reproducibility (n = 5)			
	Lauric	**Palmitic**	**Stearic**	**Linoleic**	**Lauric**	**Palmitic**	**Stearic**	**Linoleic**
5 mg/ml	6.07	8.98	7.14	7.74	9.01	8.45	7.55	13.74
20 mg/ml	1.01	4.55	5.27	6.41	3.60	4.68	5.55	6.95

* Data are given as relative standard deviation.

DISCUSSION

Lipids (fatty acids) are essential materials in newborn feeding and play an important role in nervous system development (9, 10). As the only dietary intake of the newborn is milk, it is of particular interest to qualify the intake of fatty acids by the newborn. In this context, it is important to determine the levels of some fatty acids present in infant formulas.

The methods of Gerber and Rose-Gottlieb (11, 12) are two standard extraction methods for quantitative determination of milk fats. Usually the maximum amount of fat that can be extracted by these methods is 0.3-0.4 g. In this study at least 0.5 g fat was needed to prepare free fatty acid solutions and sometimes to repeat the process, therefore these methods could not be used. We also tested Soxhlet method for fat extraction from milk powder but again insufficient fat was obtained. So the method that is described under experimental section was used. The extracted fat was clear and 12 g of fat was extracted from 50 g milk powder (83% of total milk fat).

Gas chromatography is the most current method in fatty acids analysis (6). This method has many advantages, such as high degree of separation of components with high structural similarity. It is also capable to analyze very little amounts of samples (0.1-50 µl). Accuracy, reproducibility and speed are other advantages. In order to be more volatile, usually fatty acids analyzed by gas chromatography are first esterified (6). Fatty acids can also be analyzed in non-esterifies forms (without derivatization). Using the method of direct analysis of fatty acids that avoids esterification can save time and money and reduces the probability of error. In the present study, polar column was used to analyze fatty acids in free forms. According to statistical calculations, the present method possesses adequate accuracy and precision and can be used as a practical way for the analysis of infant formula's fatty acid.

As seen in Table 1 the obtained experimental values of lauric and linoleic fatty acid contents from twenty infant formulas were in good agreement with the values proposed by standards of codex alimentary and Institute of Standard Industrial Research of Iran (I.S.I.R.I) (13, 14). In conclusion, the method validation parameters show that the procedure is sensitive, accurate, reliable and adequate for measuring free fatty acids. The obtained experimental values of lauric and linoleic fatty acid contents from twenty infant formulas were in good agreement with the values proposed by standards of codex alimentary and Institute of Standard Industrial Research of Iran.

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Conflict of interests

The authors declare that they have no competing interests.

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