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DETERMINATION OF RIBOFLAVIN CONTENT IN INFANT FORMULAS FROM SERBIA BY LIQUID CHROMATOGRAPHY WITH FLUORESCENCE DETECTION[†]

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Abstract. *As riboflavin (vitamin B_2) is essential for normal infant growth, the levels of this vitamin in different infant formulas have to be controlled. Therefore, in this study, a simple and accurate HPLC-FLD method for assessment of vitamin B_2 content in infant formulas (linearity range=0.02−2.0 μg/mL; LOQ=0.033 μg/mL; accuracy>96%) was described and validated. Ten best-selling infant formulas on Serbian market were assayed for riboflavin content employing the described method. Significant differences were found in the vitamin B_2 levels (from 68.60 to 131.18 μg per 100 mL of prepared milk) in formulas produced by different manufacturers. The values obtained for riboflavin content in all samples mainly correspond to the declared amounts by manufacturer. The conducted quality monitoring revealed that infant formulas from Serbia comply with the nutritional needs of infants with regard to vitamin B_2.*

Key words: *riboflavin, infant formula, high-performance liquid chromatography, fluorescence detection, quality monitoring*

1. INTRODUCTION

Breast milk is considered as an ideal source of nutrition and immunological support for infants. The World Health Organization strongly supports breastfeeding for newborns from birth until six months postpartum as a global public health recommendation. Sometimes, mothers are unable to make enough milk for the infants feeding and in such a case, milk-based infant formulas might be used as a supplement to breast milk or as a

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substitute if breastfeeding is impossible. Human milk provides the normative standard for infant nutrition with its unique composition that differs from infant formulas, although nutrient composition of infant formulas generally have similar functional properties as breast milk (Goedhart and Bindels, 1994; Jardi Pinana et al., 2015; Picciano, 2001).

Infant formula is a product specifically manufactured to satisfy, by itself, the nutritional requirements of infants. It is made from any combination of milk, soy, rice, whey, hydrolyzed protein, starch, amino acids and other ingredients (such as vitamins) which have been proven to be suitable for infant feeding. Nowadays, a wide variety of infant formulas are available on the market, so their nutritional safety and adequacy should be scientifically demonstrated to support normal growth and development of infants, especially during the first six months of life. Infant formula is not officially a pharmaceutical product, though in many cases the manufacturers are pharmaceutical companies. Regulatory bodies around the world agree that continual nutrient analysis, especially of vitamins and minerals, is crucial to infant formulas quality control. The nutritional composition of all infant formulas has to fulfill the global standards recommended by the European Society for Pediatric Gastroenterology, Hepatology and Nutrition's (ESPGHAN) international expert group that was commissioned by The Codex Alimentarius Commission. These documents contain data about minimum and maximum values of nutrient contents in infant formulas with the goal to provide safe and nutritionally adequate products that meet the nutritional requirements of healthy babies during the first months of life until the introduction of appropriate complementary feeding (Koletzko et al., 2005; Powers, 1997).

Riboflavin (7,8-dimethyl-10-(1'-D-ribityl)isoalloxazine, vitamin B_2), acts as a precursor and integral part of two coenzymes: flavin adenine dinucleotide (FAD) and flavin mononucleotide (FMN), which participate in oxidation–reduction reactions in many metabolic pathways (Fig. 1). As FAD is part of the respiratory chain, riboflavin is central to energy production. It is essential for development of infant tissues, the function of the muscles, the heart, the nervous system and mental activity. Also, it is involved in carbohydrate, lipid and protein metabolism, red blood cell formation, respiration, antibody production and normal infant growth. The appropriate level of this vitamin in the infant formulas is very important for infants during the first six months of life, especially for pre-term or low birth weight (Böhles, 1997; Gartner et al., 2005; Powers, 2003).

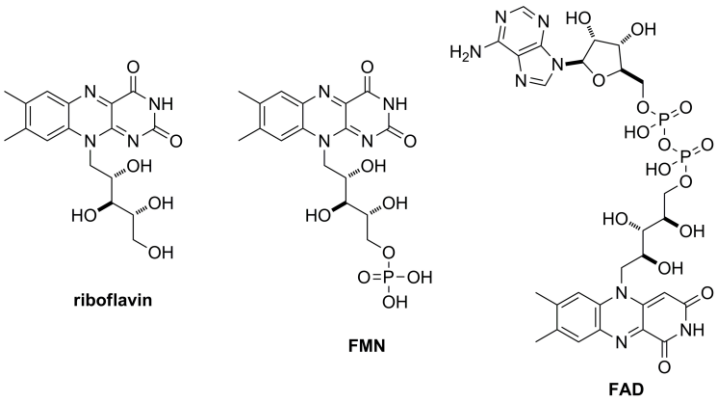

Fig. 1 Structures of riboflavin and riboflavin-containing coenzymes

A number of techniques have been proposed in the literature for the determination of riboflavin content in pharmaceutical products, biological and food samples and dietary supplements. These include microbiological and immunoassays, spectrophotometric, spectrofluorimetric, electrochemical, capillary zone electrophoresis and high-performance liquid chromatography (HPLC; either by UV or fluorescence detection (FLD)) methods (Albalá-Hurtado et al., 1997; Gao et al., 2008; Gatti and Gioia, 2005; Gliszczyńska-Świglo and Koziolowa, 2000; Jedlicka and Klimes, 2005; Sunarić et al., 2012; Viñas et al., 2004). Both microbiological and fluorometric methods are official according to AOAC International (Woollard and Indyk, 2002). Although very sensitive, microbiological assays are mostly tedious and time-consuming, and therefore HPLC−FLD methods, based on the strong natural fluorescence of riboflavin, have been the primary methods used to determine published riboflavin content of the food supply.

To the best of our knowledge, the monitoring of quality of infant formulas in Serbia with respect to the levels of vitamin B_2 has never been carried out so far. Therefore, in the present work, determination and verification of riboflavin content, using HPLC−FLD method, in some commercial brands of infant formulas available on Serbian market were reported.

2. Material and Methods

2.1. Chemicals and reagents

Standard of riboflavin, ammonium acetate (NH_4OAc), potassium carbonate (K_2CO_3), potassium hydroxide (KOH) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Hydrochloric acid (HCl), perchloric acid ($HClO_4$) and methanol (MeOH) were supplied by Merck (Darmstadt, Germany). All chemicals used were of analytical grade, with exception of MeOH that was of HPLC grade, and were used as received without any further purification. Deionized water was generated using a Smart2Pure Ultrapure water purification system from Thermo Electron LED GmbH (Niederelbert, Germany).

2.2. Apparatus and chromatographic conditions

Chromatographic determination of riboflavin was performed using a 1200 Series Agilent Technologies HPLC system (Santa Clara, CA, USA) with a binary pump, solvent degasser system and fluorescence detector. The Agilent ChemStation program was used for the monitoring of the analyses and for data acquisition and evaluation. Chromatographic separation was achieved at 30 °C using the ZORBAX Eclipse Plus C18 Solvent Saver Plus column (3.0 mm × 150 mm, 3.5 μm; Agilent Technologies, Santa Clara, CA, USA). Isocratic elution was carried out with mobile phase, consisted of 32% MeOH and 68% 5mM NH_4OAc (pH 5.0). The flow rate was kept at 0.500 mL/min and the injection volume was 20 μL. Fluorescence detector was set at λ_{ex}=440 nm and λ_{em}=520 nm and the total time of single chromatographic run was below 5 min.

2.3. Preparation of standard solutions and calibration curves

The stock standard solution of riboflavin (10 μg/mL) was prepared by dissolving the accurately weighted mass of riboflavin in 5% HCl (w/w) and stored at 4 °C in a dark

glass bottle until further use. The working standard solutions of vitamin B_2 (in concentration range from 0.02−2.00 μg/mL) were freshly prepared by diluting the stock standard solution with deionized water. Concentrations of this vitamin in the analyzed infant formulas were determined by the calibration curve method. The calibration curve was constructed by plotting the peak area of analyte against its concentration. The range of calibration curve was chosen to cover the expected concentrations of riboflavin in the analyzed samples after extraction procedure. After HPLC analysis, considering all steps in sample preparation procedure, concentrations of riboflavin were calculated and expressed as micrograms (μg) of B_2 per 100 mL of prepared milk, as given on the label. For validation, all experiments were carried out five times under identical experimental conditions.

2.4. Sample preparation

Ten most commonly used standard infant formulas were purchased from local pharmacies. All analyzed brands were initial milk-based formulas designed for infant consumption during the first six months of life. The infant formulas were dissolved and prepared following the manufacturer's instructions in order to attain vitamins concentrations as given on the label (as micrograms (μg) of B_2 per 100 mL of prepared milk). Ten grams of powder was weighed and transferred to a plastic conical tube and 25 mL of freshly boiled and cooled to 70 ºC water was added. Then, the content of the tube was vigorously shaken on vortex for several minutes. After complete dissolution and cooling of the prepared milk sample to room temperature (about 25 °C), further sample preparation for HPLC analysis was carried out.

The samples preparation procedure and riboflavin content analysis were done according to our previously published methods with some modifications (Sunarić et al., 2012). Five hundred microliters aliquot of the freshly prepared milk was treated with $HClO_4$ (12%, w/w) in the volume ratio 1:1, mixed on vortex (1 min) and allowed to stand for 15 min at the room temperature protected from the light. The protein fraction was precipitated by centrifugation at 4000 rpm for 10 min at 4 °C. Then, approximately 30 μL of 2M K_2CO_3 in 6M KOH was added to 700 μL of supernatant in order to adjust the pH value of the sample to 5.0. After vortex mixing for 1 min, the resulting insoluble perchlorate was removed by the second centrifugation (10 min, 4000 rpm at 4 °C). An aliquot of the obtained supernatant was filtered through the Syringe Econofilter (25/0.45 μm RC, Agilent Technologies, Santa Clara, CA, USA) and then 20 μL was injected into the column. All samples were analyzed in triplicate.

3. RESULTS AND DISCUSSION

3.1. Validation of HPLC-FLD method

Compared to our previously published protocol for the evaluation of the riboflavin content in different types of milk and non-dairy substitutes (Sunarić et al., 2012), the main modifications of the herein described method are (*i*) in the sample preparation procedure that is fully adapted to infant formulas (smaller volumes of the initial sample, deproteinizing agent and pH adjustment reagent), (*ii*) in the composition of the mobile phase (percentage of MeOH is lowered from 36% to 32%), and (*iii*) in the type of the chromatographic column, i.e. the so-called "Solvent Saver" C18 column is used that is

shorter and enables successful separation and quantification at lower flow rates of the mobile phase. Therefore, the validation of this new protocol was performed as well.

The parameters for method validation, such as linearity, accuracy, precision, limits of detection and quantification, were calculated after the optimum conditions for chromatographic determination with fluorescence detection were established. Linearity of the method was evaluated by regression analysis after construction of calibration curve using a series of vitamin B_2 standard solutions (concentration levels in the range of analytical interest). Linearity was observed over riboflavin concentration from 0.02−2.0 μg/mL with the correlation coefficient of 0.993.

The accuracy of the method was expressed as recovery of the extraction procedure. Recoveries were determined by the standard addition method at three concentration levels. The obtained values for the spiked samples (prepared according to the manufacturer's instructions) were 96.2%, 98.2% and 98.5%.

To study the precision of the modified method, both intra- and inter-day precisions were calculated as relative standard deviation (RSD) for three replicates of three standard solutions. The obtained results were in the range of 0.54%−0.96% and 0.98%−2.11% for intra- and inter-day precision, respectively, which indicated good precision.

The limits of detection (LOD) and quantification (LOQ) were calculated using the following equations: LOD = 3.3 x E_a/b and LOQ = 10 x E_a/b, where E_a is the standard error of the intercept, and b is the slope of the calibration curve. The obtained results for these parameters are given in Table 1.

3.2. Riboflavin content in infant formulas

The above-described and validated HPLC-FLD method was successfully applied for determination of riboflavin content in 10 best-selling infant formula brands on Serbian market. The chromatograms obtained for riboflavin standard solution at concentration of 0.04 μg/mL and one of the analyzed infant formulas (Brand 1) are shown in Fig. 2. Well separated and symmetric peaks of riboflavin were obtained at approximately 3.2 min in both samples.

Table 1 Characteristic parameters for the riboflavin calibration curve

Parameters	Values
Number of points	14
Linear range (μg/mL)	0.02−2.0
Intercept ± S.E.	1.95 ± 1.48
Slope ± S.E.	444.02 ± 7.67
Correlation coefficient	0.993
LOD (μg/mL)	0.011
LOQ (μg/mL)	0.033

The results of HPLC−FLD monitoring of the riboflavin content in the infant formulas are given in Table 2. The values are expressed as mean value of three measurements ± standard deviation (SD). It was found that the concentration of riboflavin was in the range from 68.60 to 131.18 μg per 100 mL of prepared milk. The level of this vitamin considerably varies within the studied brands of infant formula and this most likely depends on the formulation and recipe of the manufacturer. The determined concentrations of vitamin B_2 in the analyzed samples accounted for over than 88.9% of the values declared on the product label. Thus, it

could be stated that in all studied infant formulas from Serbia the content of vitamin B_2 largely corresponds to the declared values listed on packaging by the manufacturer.

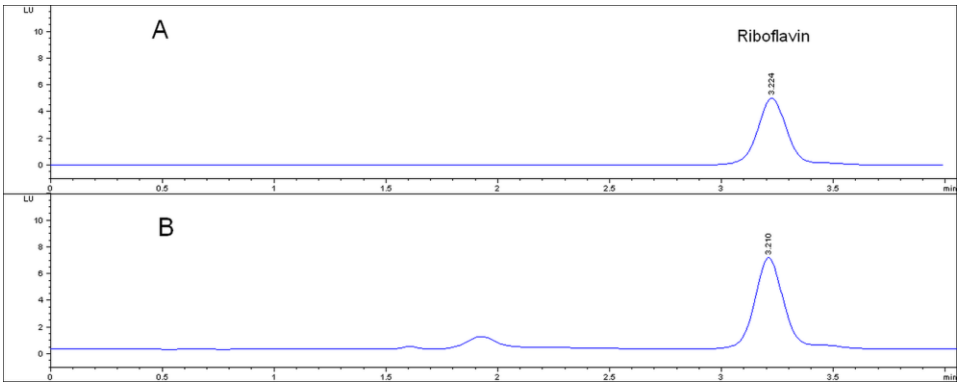

Fig. 2 HPLC-FLD chromatograms obtained for riboflavin standard solution (0.04 μg/mL; A) and one of the analyzed infant formulas (Brand 1; B)

Riboflavin is one of the more variable nutrients in infant formulas because of the differences in inherent levels in various ingredients and the effects of form and package on stability, so its concentration varies in a wide range in up to now analyzed infant formulas available in Europe (73−155 μg/100 mL) and the United States of America (78−186 μg/100 mL). According to the Ordinance on the sanitary safety of dietary products of the Republic of Serbia (the Official Gazette of RS, No. 45/10) and the regulative of the European Society for Pediatric Gastroenterology, Hepatology and Nutrition (ESPGHAN), the minimum recommended concentration of riboflavin in infant formulas should be 48 μg per 100 mL of prepared milk (i.e. 80 μg/100 kcal) (Jardi Pinana et al., 2015; Koletzko et al., 2005; MacLean Jr. et al., 2010). As we found that the content of riboflavin is within the range of 68.60−131.18 μg/100 mL, it can be concluded that all analyzed infant formulas from Serbia contain riboflavin in the amounts greater than the recommended minimum amount and completely comply with the requirements of the national and European guidelines.

Table 2 Content of riboflavin in the infant formulas available on Serbian market and the comparison of the obtained values with the contents declared by manufacturers

Infant formula	Determined content*	Declared content*	Recovery (%)
Brand 1	68.60 ± 0.57	72.0	95.3
Brand 2	81.74 ± 0.78	85.0	96.2
Brand 3	95.40 ± 0.77	100.0	95.4
Brand 4	93.34 ± 0.93	105.0	88.9
Brand 5	88.06 ± 0.64	95.0	92.7
Brand 6	113.50 ± 1.05	125.0	90.8
Brand 7	97.30 ± 0.92	100.0	97.3
Brand 8	88.73 ± 0.64	95.0	93.4
Brand 9	131.18 ± 1.14	140.0	93.7
Brand 10	80.64 ± 0.68	90.0	89.6

* In μg of riboflavin per 100 mL of prepared milk

Recommended Dietary Intake (RDI) for infants (0-6 months old) is 300 μg of riboflavin per day (Koletzko et al., 2005; Powers, 1997). Thus, assuming that the advised daily intake of 500 mL of prepared infant formula is optimal for 0- to 6-month infants, all analyzed samples supply much higher levels than the values established by the RDI for vitamin B_2 (from 114.0% to 219.7%).

3. CONCLUSIONS

The first year of life of the newborn is the period of intense growth and development. In the cases when mothers have low production of breast milk, or in the absence of mother's milk for nutrition, the usage of infant formulas is the only alternative. Although the composition of infant formulas is not completely identical to mother's milk, their composition has to satisfy very stringent international and domestic regulations deriving from the extensive and lengthy research whose principal aim was to design new formula that is even closer to breast milk than it already is. Also, the mentioned regulations strongly recommend the continuous monitoring of the quality and safety of the infant formulas available on the market.

Herein, a simple and accurate HPLC-FLD method for the evaluation of the content of vitamin B_2 in the infant formulas was described and validated. This protocol involves an acid hydrolysis and extraction of the supernatant, without enzymatic treatment. Employing this method, the monitoring of riboflavin content in the best-selling initial milk formulas available in Serbian pharmacies was carried out for the very first time. The results showed that the content of riboflavin in all infant formulas largely corresponds to the declared values of the manufacturer on the packaging. Also, the content of riboflavin in all samples exceeds the value of the PDU for infants from 0-6 months of age, so these infant formulas could be used as an adequate supplementation or replacement for breast milk.

REFERENCES

Albalá-Hurtado, S., Veciana-Nogués, M.T., Izquierdo-Pulido, M., Mariné-Font, A., 1997. J. Chromatogr. A, 778, 247–253. doi:10.1016/S0021-9673(97)00387-7

Böhles, H., 1997. Int. J. Vit. Nutr. Res., 67, 321–328.

Gao, Y., Guo, F., Gokavi, S., Chow, A., Sheng, Q., Guo, M., 2008. Food Chem., 110, 769–776. doi:10.1016/j.foodchem.2008.03.007

Gartner, L.M., Morton, J., Lawrence, R.A., Naylor, A.J., O'Hare, D., Schanler, R.J., Eidelman, A.I., 2005. Pediatrics, 115, 496–506. doi:10.1542/peds.2004-2491

Gatti, R., Gioia, M.G., 2005. Anal. Chim. Acta, 538, 135–141. doi:10.1016/j.aca.2005.02.025

Gliszczyńska-Świglo, A., Koziolowa A., 2000. J. Chromatogr. A, 881, 285–297. doi:10.1016/S0021-9673(00)00200-4

Goedhart, A.C, Bindels, J.G., 1994. Nutr. Res. Rev., 7, 1–23. doi:10.1079/NRR19940004

Jardi Pinana, C., Aranda Pons, N., Bedmar Carretero C., Arija Val V., 2015. An. Pediatr., 83, 417–429. doi:10.1016/j.anpede.2015.10.006

Jedlicka, A., Klimes, J., 2005. Chem. Pap., 59, 202–222. doi:10.1002/chin.200542295

Koletzko, B., Baker, S., Cleghorn, G., Neto, U.F., Gopalan, S., Hernell, O., Hock, Q.S., Jirapinyo, P., Lonnerdal, B., Pencharz, P., Pzyrembel, H., Ramirez-Mayans, J., Shamir, R., Turck, D., Yamashiro, Y., Zong-Yi, D., 2005. J. Pediatr. Gastroenterol. Nutr., 41, 584–599. doi:10.1097/01.mpg.0000187817.38836.42

MacLean Jr., W.C., Van Dael, P., Clemens, R., Davies, J., Underwood, E., O´Risky, L., Rooney, D., Schrijver, J., 2010. J. Food Comp. Anal., 23, 44–53. doi: 10.1016/j.jfca.2009.07.008

Ordinance on the sanitary safety of dietary products, Official Gazette of RS, No. 45, 2010.

Picciano, M.F., 2001. Pediatr. Clin. North Am., 48, 53–67. doi:10.1016/S0031-3955(05)70285-6

Powers, H., 1997. Nutr. Res. Rev., 10, 1–23. doi:10.1079/NRR19970003
Powers, H., 2003. Am J. Clin. Nutr., 77, 1352–1360.
Sunarić, S., Denić, M., Kocić, G., 2012. Ital. J. Food. Sci., 2012, 24, 352–357.
Viñas, P., Balsalobre, N., López-Erroz, C., Hernández-Córdoba M., 2004. J. Agr. Food Chem., 52, 1789–1794. doi:10.1021/jf030756s
Woollard, D.C., Indyk, H.E. 2002. J. AOAC Int., 85, 945–951.

ODREĐIVANJE SADRŽAJA RIBOFLAVINA U POČETNIM MLEČNIM FORMULAMA IZ SRBIJE KORIŠĆENJEM TEČNE HROMATOGRAFIJE SA FLUORESCENTNOM DETEKCIJOM

S obzirom da je riboflavin (vitamin B_2) neophodan za normalan rast odojčadi, sadržaj ovog vitamina u različitim infant formulama treba biti kontrolisan. Stoga, u ovom radu, razvijena je i validirana jednostavna i precizna HPLC-FLD metoda (visoko efikasna tečna hromatografija sa fluorescentnom detekcijom) za određivanje sadržaja vitamina B_2 u infant formulama (opseg linearnosti=0,02−2,00 μg/mL, LOQ=0,033 μg/mL, tačnost>96%). Deset najprodavanijih infant formula na srpskom tržištu je ispitano na sadržaj riboflavina koristeći opisanu metodu. Pronađene su značajne razlike u sadržaju vitamina B_2 (od 68,60 do 131,18 μg u 100 mL pripremljenog mleka) u formulama različitih proizvođača. Dobijene vrednosti za sadržaj riboflavina u ispitivanim uzorcima uglavnom odgovaraju deklarisanim vrednostima datim od strane proizvođača. Sprovedena kontrola kvaliteta je pokazala da su infant formule u Srbiji u skladu sa potrebama u ishrani novorođenčadi, kada se govori o vitaminu B_2.

Ključne reči: *riboflavin, adaptirano mleko za bebe, tečna hromatografija visokih performansi, fluorescentna detekcija, praćenje kvaliteta*