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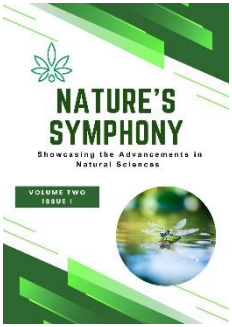

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Research Article

HPLC-Based Quantification of Metronidazole Levels in Breast Milk of Post-Cesarean Mothers

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Metronidazole, breast milk, HPLC, pharmacokinetics, maternal medication

ABSTRACT

In the present context of Nepal, 80% of birth occurs through cesarean delivery. To prevent anaerobic infections, mothers who have undergone cesarean delivery are given metronidazole. Short-term metronidazole medication might cause adverse responses and minor side effects, such as nausea and digestive problems, foul taste, tongue furring, dizziness, drowsiness, and small skin rashes. Pharmacokinetically, breast milk is supposed to be a separate compartment into which the drug is excreted-mainly by passive diffusion. The main objective of this research was monitoring of metronidazole in the breast milk of mothers who has undergone cesarean delivery. Breast milk samples were collected from 20 lactating mothers who were prescribed Metronidazole, with concentrations measured at 1.5 hours and 4.5 hours post-dose using High-Performance Liquid Chromatography (HPLC). The mean Metronidazole concentration in breast milk was found to be 7.09 mg at 1.5 hours and 6.82 mg at 4.5 hours. The study further examined the relationship between maternal factors, including delivery history and concurrent medication use, on Metronidazole concentration, revealing that first-time mothers had higher concentrations in breast milk compared to mothers with multiple deliveries. Blood samples from infants were also analyzed to assess metronidazole concentrations, and a significant positive correlation ($r = 0.9449$, $p < 0.0001$) was observed between infant body weight and drug levels in the blood. The study emphasizes the importance of considering these factors when prescribing medications to breastfeeding mothers to ensure infant safety. Although Metronidazole is considered compatible with breastfeeding by many guidelines, the results suggest that factors such as maternal history, concurrent drug use, and infant weight can influence the extent of drug exposure in breast-fed infants. These findings underscore the importance of personalized medical advice for breastfeeding mothers who are prescribed Metronidazole or other medications.

1. INTRODUCTION

Milk is the primary source of nutrition for newborns before they are able to eat and digest other foods. The World Health Organization (WHO) recommends exclusive breastfeeding for the first six months of life, with solids gradually being introduced around this age when signs of readiness are shown. The advantages of human milk for the optimum growth and development of infants have become more widely recognized, and as a

result, there has been an increase in the incidence and duration of breastfeeding. Although the excretion of drugs into breast milk has been of interest for several years, the increased use of breast milk provides an added incentive for obtaining a better understanding of the transfer of drugs into breast milk. This stems from the fact that the passage of significant amounts of these agents into nursing infants may affect their health and safety (Marson *et al.*, 2013). Pharmacokinetically, breast milk is supposed to be a separate compartment into which the drug is excreted-mainly by passive diffusion (Blanchard *et al.*, 1990; Kmetec and Roškar, 2003). Among the many agents studied, Metronidazole is known to be consumed by mothers undergoing cesarean delivery to prevent bacterial infection. Metronidazole is present in human milk at concentrations like maternal serum levels. Studies suggest that milk and blood samples showed almost identical levels of metronidazole metabolite (Heisterberg and Branebjerg, 1983). Because of the potential for tumorigenicity shown for metronidazole in mouse and rat studies, a decision should be made whether to discontinue nursing or to discontinue the drug, taking into account the importance of the drug to the mother; alternatively, a nursing mother may choose to pump and discard human milk for the duration of metronidazole therapy, and for 24 hours after therapy ends and feed her infant stored human milk or formula.

Mature human milk has a fat content of 3% to 5%, a protein content of 0.8% to 0.9%, a lactose content of 6.9% to 7.2%, and a mineral content of 0.2% expressed as ash. It has 60–75 kcal of calories per 100 ml. Colostrum's protein concentration is significantly higher and its carbohydrate content is lower than that of mature milk (Jenness, 1979). Although there are significant nocturnal changes and an increase in fat content with each nursing session, the fat content does not change consistently during lactation. There isn't much of a variation in the composition of milk between the two breasts unless one is infected, regardless of race, age, or nutrition (Gross *et al.*, 1981).

High-performance liquid chromatography is a technique in analytical chemistry used to separate, identify, and quantify each component in a mixture. HPLC relies on the pressure of mechanical pumps on a liquid solvent to load a sample mixture onto a column, in which the separation occurs. A HPLC separation column is filled with solid particles (e.g. silica, polymers, or sorbents), and the sample mixture is separated into compounds as it interacts with the column particles. HPLC separation is influenced by the liquid solvent's condition (e.g. pressure, temperature), chemical interactions between the sample mixture and the liquid solvent (e.g. hydrophobicity, protonation, etc.), and chemical interactions between the sample mixture and the solid particles packed inside of the separation column (e.g. ligand affinity, ion exchange, etc.). A typical HPLC consists of: - a pump, a reservoir, an injector, a detector, and data handling device.

2. METHODOLOGY

2.1 Study Design

A cross-sectional descriptive study was done.

2.2 Study Setting

The study was carried out in Nepal Medical College, Jorpati, Attarkhel. The study was started at 28th October 2019 and ended on 25th December.

2.3 Study Population and Sample

The study was performed on twenty mothers on the basis of the availability of samples and the time limitation of the research study.

2.4 Inclusion Criteria

- The mother underwent cesarean delivery and Metronidazole therapy.
- Sample collection after the third day of delivery.

2.5 Exclusion criteria

- Mothers undergone normal delivery.
- Mothers having health problems.
- Mothers who denied consent and participation.

2.6 Sampling Procedures

- Purposive sampling was done.
- Samples (breast milk) were collected from a total of twenty mother's undergone cesarean delivery. From each participant (mothers), breast milk was collected at 1.5 hours and 4.5 hours after the administration of Metronidazole. All the samples from each mother were extracted using a breast pump and collected in a vial, labeled with sample numbers and intervals. All the collected samples were kept in the icebox and brought safely to the college laboratory.
- Data collection tools: the semi-structured interview was taken from each participant.

2.7 Sample Pretreatment

Samples taken at two different intervals (1.5 hours and 4.5 hours) were deproteinized (Passmore et al.). Deproteinization was carried out by making the equilibrium mixture of methanol and acetonitrile (20ml: 20ml) and by mixing an equal amount of this mixture and milk samples (6ml mixture and 6ml milk) in a centrifugation tube. Samples were centrifuged in a centrifugation tube at 500rpm for 10-15mins. Then the supernatant part (2ml) was carefully transferred to the test tube.

2.8 Mobile Phase

The mobile phase for HPLC analysis was prepared by using 7%acetate buffer pH4 and acetonitrile in the ratio 7:93. Acetate buffer was prepared by mixing 0.57ml glacial acetic acid and 0.1gram sodium hydroxide and making volume up to 200ml with HPLC grade water and adjusting pH to 4 (Salvesen *et al.*, 1984).

2.9 Standard Preparation

Blank milk was used as medium for preparing standard curves to which a known amount of Metronidazole was added to yield five different concentrations (i.e. 6.67ppm, 10ppm, 13.33ppm, 14.28ppm, 15ppm). Blank milk was used to prepare the calibration curves in order to mimic the actual sample matrix as closely as possible. The blank milk was obtained from a nursing mother undergoing normal delivery.

2.10 High Performance Liquid Chromatography Conditions

The HPLC system was operated at a temperature of 35ºC with peak detection at a wavelength of 320 nm. The flow rate was maintained at 2mL/min.

2.11 Data Collection Tools\Instruments

2.11.1 List of Materials

The active ingredient as standard and other chemicals are shown in Table 1.

2.12 Ethical Consideration

IRC letter with reference number:005-076/077 was received from Nepal Medical College, Attarkhel, Jorpati-7.

Table 1. List of materials

Materials	Source
Metronidazole (active)	Aadee Remedies Pvt. Ltd.
Acetic acid	CIST college
Sodium hydroxide	CIST college
HPLC grade water	CIST college
HPLC grade acetonitrile	CIST college
HPLC grade methanol	CIST college

3. RESULTS

This section provides a detailed description of the results from the analysis done by using HPLC. The variables are described as a simple percentage, pie chart, bar diagram, and frequency table as appropriate.

3.1 Results on the Concentration of Metronidazole in Mothers' Breast Milk

3.1.1 Standard Calibration Curve

High-Performance Liquid Chromatography (HPLC) was used to create a standard calibration curve to measure the amounts of metronidazole in breast milk samples. By diluting the stock solution with the proper solvent, a number of metronidazole standard solutions with known concentrations were created. The range of values utilized was 5 ppm to 20 ppm for identifying the anticipated amounts of metronidazole in breast milk. Preliminary method development experiments were used to determine the proper mobile phase, flow rate, and detection wavelength before each standard solution was injected into the HPLC system under optimal circumstances. Metronidazole-corresponding peak regions were measured, and the chromatograms were recorded. The method's suitability for quantification was confirmed by the observation of a linear connection between the peak area and the concentration of metronidazole. The calibration curve was draw, the slope obtained is y = 30.565x + 57.221 and R*R=0.9994.

3.1.2 Concentration of Metronidazole in Breast Milk

Breast milk samples were collected from 20 mothers those were given metronidazole and

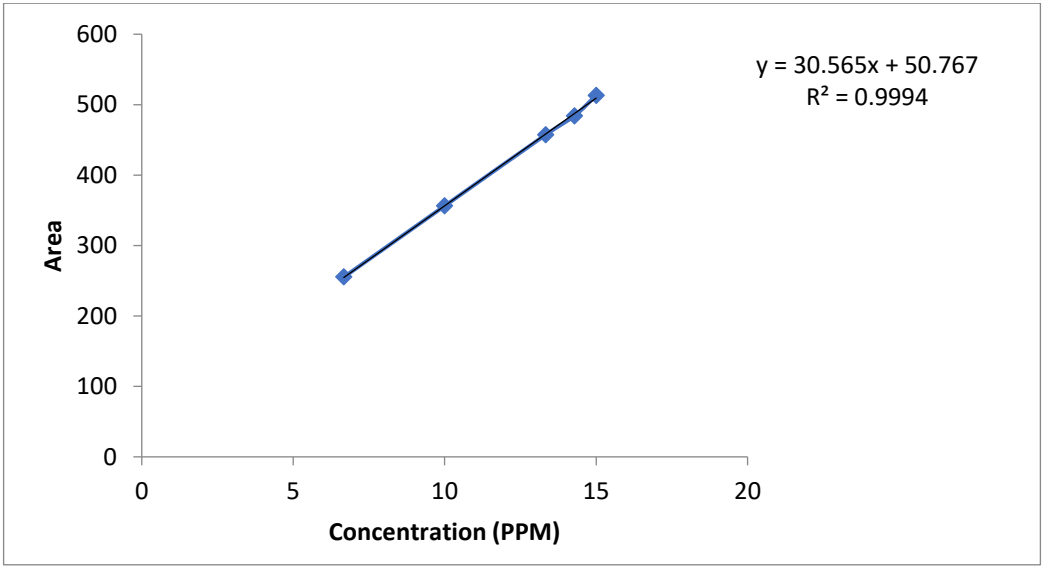

Figure 1. Standard calibration curve

samples were collected after 1.5 hours and 4.5 hours after metronidazole administration to estimate the potential exposure to the infant. From different sources, it was found that the average milk needed or fed by a baby is 0.8 to 1 ounce a day and about 14-22 times a day for a 3 day old baby, so the minimum volume of milk intake by the baby is 360ml. Metronidazole was prescribed to the mothers for a course of 7 days, we assumed that the baby would consume this consistent daily volume (360 mL) throughout the duration of treatment.

Table 2 shows the individual Metronidazole concentrations in breast milk for each participant at 1.5 hours and 4.5-hours post-administration. The daily potential exposure of the infant to Metronidazole through breast milk was calculated by averaging the concentrations from the two time points and then multiplying this average by the daily milk intake (360 mL).

Metronidazole is given for 7 days, assuming the baby's feed is 360ml only for 7 days, the minimum and maximum concentrations of metronidazole the baby obtained are summarized in Table 4.

Table 2. Concentration of Metronidazole in 1.5hrs and 4.5 hrs (in ppm)

S.N.	Concentration of Metronidazole in 1.5hrs (in ppm)	Concentration of Metronidazole in 4.5hrs (in ppm)
P1	16.94	26.13
P2	21.64	14.42
P3	30.50	7.27
P4	7.12	26.57
P5	11.92	13.96
P6	20.48	17.87
P7	53.26	44.10
P8	29.32	34.53
P9	17.58	45.60
P10	16.33	30.38
P11	27.97	13.94
P12	37.59	11.57
P13	13.46	13.46
P14	10.52	9.52
P15	35.10	9.76
P16	12.03	12.66
P17	13.82	11.15
P18	8.86	17.98
P19	4.90	10.58
P20	4.76	7.70

Table 3. Concentration of Metronidazole determined

S. N	Concentration in mg (1.5 hr.)	Concentration in mg (4.5 hr.)	Estimated Daily Metronidazole Exposure (mg)
P1	6.1	9.41	2807.4
P2	7.79	5.19	2349.6
P3	10.98	2.62	2436.0
P4	2.56	9.57	2187.6
P5	4.29	5.03	1699.2
P6	7.37	6.43	2496.0
P7	19.17	15.87	6328.8
P8	10.56	12.43	4143.6
P9	6.33	16.42	4116.0
P10	5.88	10.94	3026.4
P11	10.07	5.02	2705.4
P12	13.53	4.17	3192.0
P13	4.84	4.84	1742.4
P14	3.79	3.43	1332.6
P15	12.64	3.51	2907.0
P16	4.33	4.56	1569.6
P17	22.91	72.06	17059.8
P18	4.77	23.48	5070.0
P19	1.76	3.81	995.4
P20	1.71	2.77	805.2

Table 4. Summary for Concentration of Metronidazole

	In ppm		In mg	
	1.5hrs	4.5hrs	1.5hrs	4.5hrs
Maximum	53.26	45.6	19.17	16.42
Minimum	4.76	7.27	1.71	2.62
Average	19.70	18.96	7.09	6.82
Standard deviation	12.53	11.68	4.51	4.20

3.2 Result on Demographic Characteristics of Mothers

3.2.1 Number of Deliveries

A correlation between the delivery history of mother and the concentration of metronidazole was studied. The study included three groups of mothers, (a) first-time mothers (primiparous), (b) mothers with a second delivery (multiparous), and (c) mothers with a third or subsequent delivery (grand multiparous). Sample was collected on same time intervals that is 1.5 hours and 4.5 hours.

It was observed that the highest concentration of Metronidazole was present in the breast milk of first-time mothers. On average, the concentration of Metronidazole in primiparous mothers was significantly higher than that observed in mothers who had undergone multiple deliveries Possible reasons for this could include differences in breast tissue **Table**

4. Average Metronidazole Concentrations by Delivery Group

Delivery Group	Average Concentration at 1.5 hr (mg)	Average Concentration at 4.5 hr (mg)
Primiparous (First Delivery)	12.3 mg	15.7 mg
Multiparous (Second Delivery)	7.6 mg	8.4 mg
Grand Multiparous (Third Delivery or more)	3.9 mg	5.1 mg

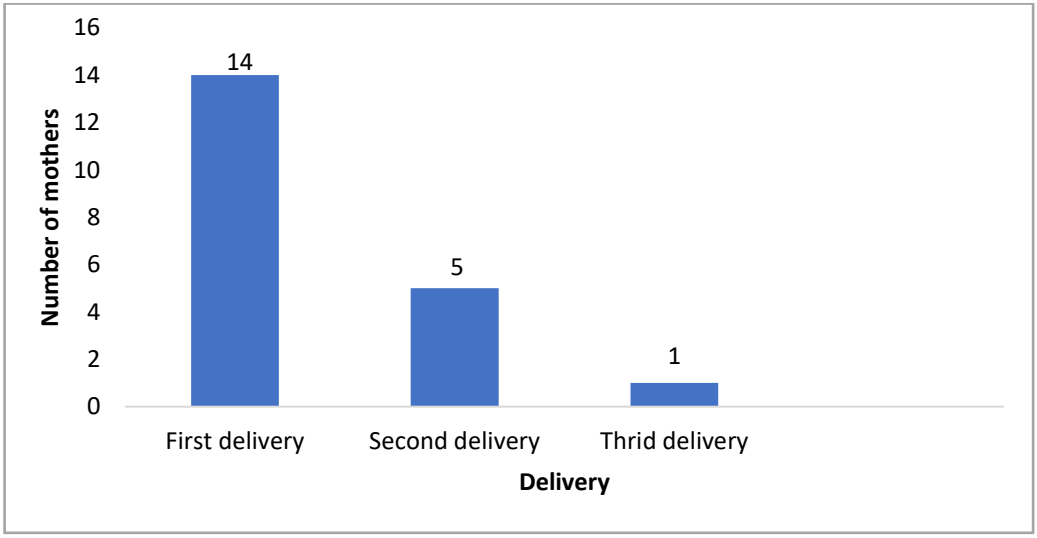

Figure 2. Number of mothers vs Number of deliveries

development, milk production regulation, and metabolism of the drug in first-time mothers. The physiological changes experienced after a first pregnancy and delivery may influence drug metabolism or milk production, leading to lower concentrations of Metronidazole being transferred to the milk.

3.2.2 Analyses of Metronidazole in Infants

In a subsequent phase of the study, blood samples from the infants of the 20 mothers who were prescribed metronidazole were collected to evaluate the drug's concentration in the infants' blood. The body weight of each infant was also recorded to assess any correlation between the infant's weight and the amount of Metronidazole found in their bloodstream.

The infants were divided into three weight categories, (a) Infants weighing ≤ 3.0 kg, (b) Infants weighing between 3.1 kg and 3.5 kg, (c) infants weighing ≥ 3.6 kg. The results revealed that infants with higher body weights tended to have higher concentrations of metronidazole in their blood, suggesting that body mass might influence how much of the drug is absorbed or retained from breast milk.

The positive correlation between infant body weight and Metronidazole concentration suggests that heavier infants might absorb or metabolize the drug differently compared to lighter infants. This finding is significant because it indicates that weight could be a factor in determining the amount of drug exposure an infant receives through breastfeeding.

Table 5. Correlation Between Infant Body Weight and Metronidazole Concentration

Infant Weight Group	Average Metronidazole Concentration in Blood (mg/L)
≤ 3.0 kg	0.15 mg/L
3.1 – 3.5 kg	0.26 mg/L
≥ 3.6 kg	0.42 mg/L

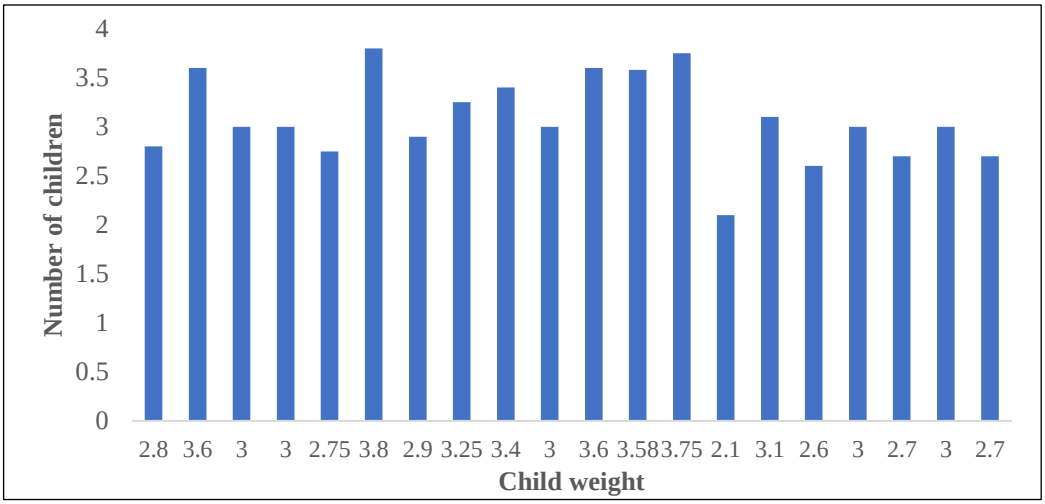

Figure 4. Number of children vs Child weight

To apply statistical analysis to the findings related to Metronidazole concentrations in infants' blood samples and their correlation with body weight, we can use several common statistical methods

Infant Weight Groups: Low (≤3.0 kg), Medium (3.1-3.5 kg), High (≥3.6 kg)

3.2.3 Mean Metronidazole Concentrations in Blood (mg/L)

- Low Weight Group: 0.15 mg/L
- Medium Weight Group: 0.26 mg/L
- High Weight Group: 0.42 mg/L

3.2.4 Pearson Correlation Coefficient

Pearson's correlation measures the strength of the linear relationship between two variables—in this case, infant body weight and Metronidazole concentration. The correlation coefficient ranges from -1 to 1:

- r=1r = 1r=1 means perfect positive correlation.
- r=0r = 0r=0 means no correlation.
- r=−1r = -1r=−1 means perfect negative correlation.

Correlation Coefficient (r): 0.9449
P-value: 3.66×10−103.66 \times 10^{-10}3.66×10−10

The Pearson correlation coefficient of 0.9449 indicates a very strong positive correlation between infant body weight and Metronidazole concentration in their blood. The p-value is extremely low, suggesting that this correlation is highly statistically significant.

3.3 Statistical Analysis

SPSS software was used for statistical analysis.

Table 6. Significance between Concentration and Sampling Days

Test Statistics		
	Conc_15	Conc_45
Chi-Square	1.339	1.006
Df	1.24	1.31
Asymp. Sig.	7	6

Table 7. Significance between Concentration and Age

Correlations					
			Conc_15	Conc_45	Age
Spearman's rho	Conc_15	Correlation Coefficient	1.000	.559*	-.045
		Sig. (2-tailed)	.	.010	.850
		N	20	20	20
	Conc_45	Correlation Coefficient	.559*	1.000	-.202
		Sig. (2-tailed)	.010	.	.393
		N	20	20	20
	Age	Correlation Coefficient	-.045	-.202	1.000
		Sig. (2-tailed)	.850	.393	.
		N	20	20	20

*. Correlation is significant at the 0.05 level (2-tailed).

Variables like concentrations, age, and maximum concentration were compared for significance. The various inferential statistics showed that the comparison between any variables was not significant.

4. DISCUSSION

The present study aimed to assess the transfer of Metronidazole into breast milk and its potential impact on breastfeeding infants. The findings show a slight increase in Metronidazole concentration in breast milk from 1.5 hours (mean: 8.07 mg) to 4.5 hours (mean: 11.08 mg), suggesting the drug persists and may accumulate slightly over time. However, statistical analysis via a paired t-test indicated that this difference was not significant ($p = 0.303$) (Altman, 1990; Howell, 1992). This lack of statistical significance could be attributed to the variability in the pharmacokinetics of Metronidazole among different individuals, which is influenced by factors such as maternal metabolism, drug clearance, and absorption dynamics (Astrup-Jensen *et al.*, 1996). Despite the lack of statistical significance between the two time points, the concentration values are relevant for understanding potential infant exposure. Studies indicate that prolonged or repeated use of drugs such as Metronidazole could lead to cumulative effects in breastfed infants, necessitating further research into long-term exposure effects. A novel aspect of this study was the exploration of how maternal factors, such as delivery history, influence

Metronidazole concentration in breast milk. This finding aligns with existing literature suggesting that metabolic processes can change with subsequent pregnancies, potentially affecting the pharmacokinetics of drugs during lactation (Dorne, 2004). These differences might be explained by physiological changes in hormone levels, mammary gland development, and milk production efficiency after multiple pregnancies. Another key finding was the strong positive correlation between infant body weight and Metronidazole concentration in their blood ($r = 0.9449$, $p < 0.0001$). This correlation indicates that heavier infants tend to have higher concentrations of Metronidazole in their blood, likely due to increased milk intake and better drug absorption. Infants with higher body weights may also have more developed gastrointestinal and hepatic systems, allowing for greater drug absorption and reduced clearance time (Cardoso *et al.*, 2023).

5. CONCLUSION

The study reported significant variability in Metronidazole concentrations in breast milk among the participants, with daily infant exposure ranging from as low as 805.2 mg to as high as 17,059.8 mg. This wide range highlights the potential for varying levels of drug exposure to infants based on factors such as maternal metabolism, timing of drug administration, and breastfeeding frequency. Further research is needed to understand the clinical implications of these varying concentrations on infant health. In this study it also indicated a clear relationship between the delivery history of the mother and the concentration of metronidazole in breast milk. It suggests a stronger or slower drug transfer mechanism in their breast milk compared to mothers with multiple deliveries. The mothers with second or subsequent deliveries showed progressively lower concentrations, potentially due to physiological adaptations that occur after successive pregnancies. The concentration of metronidazole in the bloodstream of breastfeeding infants proved that heavier infants exhibited higher blood concentrations, likely due to greater overall absorption of Metronidazole from breast milk. This could be explained by the larger body mass and higher milk intake relative to their body size, or differences in the metabolism and clearance of the drug in infants of varying weights.

Conflict of Interest

The authors declare no conflict of interest.

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