

# Bilirubin Pigments in the First Meconium of Newborn Infants

Sina Aziz, Shazia Anjum<sup>1</sup>, Syed Ata-ur-Rehman<sup>1</sup>, Durre Samin Akram<sup>2</sup>, Syed Ali Anwar Naqvi<sup>3</sup>, Syed Adibul Hasan Rizvi<sup>3</sup>  
Department of Pediatrics, Sindh Institute of Urology and Transplantation (SIUT)<sup>3</sup>, Karachi, HEJ Research Institute, University of Karachi<sup>1</sup>, Department of Pediatrics, Civil Hospital<sup>2</sup>, Karachi.

## Abstract

**Objective:** To determine the pattern of excretion of total bilirubin IX $\alpha$  and IX $\beta$  in the first meconium of newborn infants.

**Methods:** First two newborns of varying gestational age were selected every week through random sampling from the neonatal unit. Of the 41 newborn infants selected 8 expired before meconium passage, hence the results are from 33 newborns. Meconium was collected and stored at -20°C, protected by aluminium foil. Samples were defrosted, vortex mixed with equal amount of dimethyl-sulfoxide, centrifuged, and analyzed by HPLC.

**Results:** Unconjugated Bilirubin-IX $\alpha$  and -IX $\beta$  were identified and quantitative estimation of Bilirubin-IX $\alpha$  done. Bilirubin-IX $\beta$  was greater than 50% of the total, in the first meconium of the newborn. Amount of bilirubin excreted in meconium was 29.2 - 90.8 mg [0.051 - 0.155 mmol] per sample of meconium passed. Amount was 9.7 mg/Kg of body weight in term newborn and 12 mg / kg in preterm.

**Conclusion:** The amount of bilirubin -IX $\beta$  decreases and bilirubin-IX $\alpha$  increases with increasing gestational age. Newborns with birth asphyxia (BA) had significantly greater quantity of bilirubin in meconium, compared to infants without BA (JPMA 55:188;2005).

## Introduction

Meconium is a dark-black-green colour material with a viscous consistency.<sup>1</sup> It is swallowed from the amniotic fluid, together with biliary and intestinal cell debris secretions, with pH near 6.1.<sup>2</sup>

As the age of the foetus increases, the meconium moves along the intestinal tract. The first trace of meconium can be detected in the ileum at 10 to 14 weeks of fetal age and by 16 weeks meconium reaches the colon.<sup>3</sup> Anal closure in the foetus occurs at 20 weeks of gestation, henceforth, no meconium is excreted till the birth of the normal newborn. Hence, the meconium excreted during the first hours of life could reflect foetal metabolism during later gestation.<sup>4</sup>

In full-term neonates the elimination of meconium was said to occur within the first 12 hours of life in 69% of infants, 12 to 24 hours 25% and 24 to 48 hours in 6% of infants.<sup>5</sup> In very low birth weight infants, the elimination can be prolonged for as long as 6 or 7 days.<sup>6</sup> Meconium contains lipids, steroids, glycoproteins, minerals, enzymes and 80 to 180mg of bilirubin i.e., approximately 1 mg of

bilirubin per gram of wet weight of meconium.

During foetal life, blood of the umbilical-placental circulation passes through the patent ductus arteriosus and hepatic circulation to reach the caval veins.<sup>7</sup> It has been suggested that bilirubin entering foetal liver can be taken up by the foetal liver cells and might be excreted in bile contributing to colour of meconium.<sup>8</sup>

The daily production of bilirubin in the full term newborn infant is 6 to 10 mg/kg per day<sup>9,10</sup>, it is estimated, that the total bilirubin content of the excreted meconium corresponds to the quantity produced in approximately 2 to 15 days.

In adult humans and most animals, Bilirubin IX $\beta$  is the most abundant pigment. This is thought to result from the high stereo selectivity of the heme-oxygenase and of the Biliverdin IX $\alpha$  reductase, which results in the formation of Bilirubin IX $\alpha$ . The Bilirubin IX $\beta$  has been detected previously in human foetal and in Gunn rat bile. However, quantitative information was lacking because analysis was based on diazo coupling procedures.<sup>11</sup>

Intestinal reabsorption of unconjugated bilirubin

pigments in meconium. Quantitative information concerning non-alpha bilirubin isomers in humans is not comprehensive.<sup>12</sup> In this study we quantified total bilirubin in the first meconium of the newborn infant utilizing a novel HPLC based methodology. The pattern of bilirubin pigments in the first meconium was studied. Hypothesis was that Bilirubin IX $\beta$  is greater than bilirubin IX $\alpha$  in the first meconium of the newborn infant.

## Methods

Meconium was recovered daily in the neonatal care unit/nursery from newborn infants. Group 1 consisted of preterm newborn with gestational age of  $\leq 32$  weeks (lowest gestational age was 24 weeks), group 2 consisted of newborns with gestational age 33 to 36 weeks and group 3 consisted of term newborns with gestational age  $\geq 37$  weeks. The newborns were divided in these subgroups based on previous studies<sup>13-15</sup> where a difference in the bilirubin content in babies with varying gestational ages was observed.

Most of the full term neonates were breast fed, whereas the preterm neonate received parenteral nutrition during first few days. The first meconium passed by 33 newborn infants term and preterm of gestational age 24 to 40 weeks, was collected by rapidly freezing the diapers with their content. The frozen meconium was then scraped off. The frozen material was stored at  $-20^{\circ}\text{C}$  and was protected from light by aluminum foil. Just before analysis the material was defrosted and weighed. All manipulations were done in dim light.

### Storage and preparation of samples

Samples of meconium were weighed carefully and were vortex mixed thoroughly for at least 5 minutes with an equal weight of dimethyl sulfoxide (DMSO). The resulting upper organic layer was removed quantitatively and stored at  $-20^{\circ}\text{C}$  for additional analysis within a week.

### Chromatographic separation of the bilirubin<sup>13-15</sup>

High-pressure liquid chromatography (HPLC) separation was performed on a Chrom Cartridge 250/4.6 Nucleosil 5C-18 column at  $40^{\circ}\text{C}$  (model 6000: Waters Associates, Miliford MA. USA). A convex gradient of mobile phase at a rate of 1.3 mL/min started with solution A and ended after 8 minutes with solution B. Solution A consisted of methanol-sodium ascorbate (1mg/ml) - Pica reagent in a volume ratio of 65:41.5:1.3. Solution B was composed of methanol, ethanol sodium ascorbate, and pica reagent in a volume ratio of 75:10:17:1.1. Elution was continued for 6 minutes with solvent B at a flow rate of 1.3 mL/min and the column was then equilibrated for 10 minutes with solution A. The pigment in the effluent was detected at 430 nm, and the area under each peak on the chromatogram was computed with an electronic integrator.

Optimal separation of the bilirubin was obtained with solvent A at a column temperature of  $45^{\circ}\text{C}$ . Under the conditions used, the chromatogram was completed in 15 minutes. A total of 25 minutes was needed for each cycle including the 10 minutes used for the re-equilibration of the column

### Chemicals

All solvents and reagents were analytical grade. Ethanol and Methanol were acquired from AnalaR (BDH Laboratories, Poole, UK). DMSO was acquired from Merck AG (Darmstadt, Germany). Pica reagent was acquired from Water Associates. Standard bilirubin preparation of sigma was used for comparison purposes.

### Validation of extraction and HPLC procedure<sup>14</sup>

The completeness of the extraction of bile pigments from meconium was checked by adding a known amount of bilirubin-IX $\alpha$ , IX $\beta$  as reference material to a sample of meconium. Each sample was vortex mixed thoroughly at room temperature for approximately 5 to 10 minutes, and treated further as mentioned earlier. Recovery varied between 97% and 100% ( $n = 3$ : each) for bilirubin -IX $\alpha$ , and between 95 and 100% for -IX $\beta$ . The coefficient of variation was 3% for bilirubin -IX $\alpha$  and 5% for -IX $\beta$  ( $n=5$ : each).

Validation of extraction was also confirmed, each day. Injecting the reference bilirubin before and after the meconium samples into the HPLC did this. Time of appearance of reference bilirubin peak was noted. After 4 to 5 meconium samples were injected into the HPLC, reference bilirubin was re-injected to confirm same time appearance.

### Bilirubin pigments

In the meconium, only Bilirubin IX $\alpha$  and IX $\beta$  were detected. The amount of IX $\alpha$  was calculated quantitatively due to the availability of the reference bilirubin IX $\alpha$ . However the percentages of IX $\alpha$  and IX $\beta$  in the samples were calculated.

### Statistics

Results are reported as mean + SD. Student t test was used to compare the means between two groups with  $p$  less than 0.05 considered significant.

## Results

The newborns had a varied diagnosis including meconium aspiration syndrome (MAS), birth asphyxia (BA), very low birth weight infants (VLBW), sepsis, preterm newborn, RDS, aspiration pneumonia, hydrocephalus, TTN, some admitted for observation only and preterm newborn admitted with and without sepsis.

Birth asphyxia was defined as presence of foetal acidosis

**Table 1. Amount of bilirubin excreted in the first sample of meconium in term and preterm newborn**

Group	$\alpha$ -bil $\mu\text{mol}$	$\beta$ -bil $\mu\text{mol}$	$\alpha+\beta$	$\alpha \times 100$ $\alpha+\beta$	$\beta \times 100$ $\alpha+\beta$
Group I (n = 13)					
Mean $\pm$ SD	0.1 $\pm$ 0.4*	2.0 $\pm$ 1.8**	2.2 $\pm$ 1.7#	14.2 $\pm$ 25.7	85.7 $\pm$ 25.7
Range	0 - 1.32	0.1 - 06.4	0.2 - 6.4	0 - 88.6	11.4 - 100
Group II (n = 13)					
Mean $\pm$ SD	2.9 $\pm$ 4.6	7.6 $\pm$ 9.0	10.5 $\pm$ 12.9	25.5 $\pm$ 27.0	74.5 $\pm$ 27.0
Range	0 - 14.8	0.1 - 30.9	0.4 - 45.7	0 - 86.2	13.8 - 100
Group III (n = 7)					
Mean $\pm$ SD	1.3 $\pm$ 3.1	4.7 $\pm$ 6.4	6.0 $\pm$ 9.1	21.7 $\pm$ 28.0	78.3 $\pm$ 28.0
Range	0 - 1.96	0 - 12.1	0.03 - 14.1	0 - 100	0 - 100
Group I	< 32 weeks				
Group II	33 - 36 weeks				
Group III	> 37 weeks				

\* P = 0.04 (Group I vs Group II)

\*\* P = 0.04 (Group I vs Group II)

# P = 0.03 (Group I vs Group II)

$\alpha$ -bil = IX alpha bilirubin

$\beta$ -bil = IX beta bilirubin

**Table 2. Comparison of weight of meconium, Bilirubin IX $\alpha$  and IX $\beta$  in newborns with (group 1) birth asphyxia and without (group 2) birth asphyxia**

Group	Weight meconium (mg)	$\alpha$ -bil*	$\beta$ -bil**	$\alpha \times 100$ $\alpha+\beta$	$\beta \times 100$ $\alpha+\beta$
Group 1(n=19) Mean $\pm$ SD	1253 $\pm$ 1466	2.3 $\pm$ 4.1	7.0 $\pm$ 8.3	22.9 $\pm$ 24.9	77.0 $\pm$ 24.9
Group 2 (n=14) Mean $\pm$ SD	918 $\pm$ 898	0.3 $\pm$ 0.5	2.3 $\pm$ 1.9	20.3 $\pm$ 31.7	79.7 $\pm$ 31.7

\* P = 0.05 (Group I vs Group II)

\*\* P = 0.03 (Group I vs Group II)

and preterm newborn admitted with and without sepsis.

Birth asphyxia was defined as presence of foetal acidosis (pH<7.0), a 5 minute apgar score of 0-3, hypoxic ischaemic encephalopathy (altered tone, depressed level of consciousness, seizures) and other multiple organ system signs.<sup>16</sup>

Table 1 shows the excretion of total bilirubin IX $\alpha$  and IX $\beta$  ( $\mu\text{mol}$ ) in the first meconium of the newborn infant with gestational age from 24 to = 37 weeks divided into

three groups. The percentage of bilirubin IX $\alpha$  and IX $\beta$  in these three groups is also shown. Percentage of IX $\beta$  was greater than 50% of the total meconium bilirubin. In some babies no bilirubin IX $\alpha$  was detected. This is because in the newborn more of IX $\beta$  is excreted rather than IX $\alpha$ .

#### Comparison between groups of infants, with differing diagnosis

Nineteen out of the 33 babies had BA (group 1) as their first diagnosis on admission to the NICU. Fourteen of

a diverse diagnosis. Comparison between the two groups is shown in Table 2. Group 1 newborn with birth asphyxia had significantly greater quantity of UCB Bilirubin IX $\alpha$  and IX $\beta$  in their meconium, compared to group 2 without birth asphyxia.

Babies without BA had a varied diagnosis. Six of thirty three newborns (18%) had LBW, 3/33 had MAS (9%), 4/33 had VLBW (12%) and only one newborn was admitted with a primary diagnosis of TTN.

## Discussion

The newborns had a varied diagnosis similar to previous studies done in our neonatal unit.<sup>17</sup> In the current study we quantitatively-demonstrated bilirubin IX $\alpha$  in the meconium, alongwith bilirubin IX $\beta$ . The peak of IX $\alpha$  and IX $\beta$  coincided with the previous work done.<sup>13-15</sup> The amount of bilirubin excreted was greater in the term newborn with a larger body weight and increasing gestational age ( $\geq 34$  weeks). Though this study has not related the haemoglobin at birth with bilirubin, previous work done by Isherwood et al<sup>18</sup> indicates that formation of bilirubin depends upon the blood volume, haemoglobin concentration and biliary excretion rate. It is known that a higher production rate of bilirubin 6mg/kg/day is presumably due to a greater hemoglobin mass and a shorter half life of erythrocytes (82 $\pm$ 15day) and a more pronounced early labelled bilirubin fraction.<sup>19</sup>

The unconjugated bilirubin IX<sup>b</sup> was previously detected in neonatal bile.<sup>20</sup> However, quantitation, was not possible by the methods available at that time.<sup>11,21</sup> This method allows quantitation of unconjugated bilirubin IX $\beta$  and IX $\alpha$ .<sup>9-11</sup> The bilirubin IX $\gamma$  and IX $\delta$  were not detected in these samples. Other established methods done in serum,<sup>22</sup> have indicated the quantification of Bilirubin IX $\alpha$ , but not from meconium or stool. This study also suggests that UCB-IX<sup>b</sup> may also be used as a marker of meconium alike coproporphyrin as in the work of Gourley et al.<sup>23</sup>

In this study, the new aspect seen was babies with BA were compared with babies without BA; there was a significant difference in the UCB -IX $\alpha$  and -IX $\beta$ . Though an interesting observation, the sample size is too small to comment upon. Further studies are required on the UCB -IX $\alpha$  and -IX<sup>b</sup> pattern in babies admitted in neonatal units with varying diagnosis.

This study has shown that the bilirubin estimation in the faeces can be done by this method as reported earlier.<sup>15</sup> Validation and extraction of this procedure has already been documented in previous studies.<sup>13-15</sup> The method has been first time adopted in Pakistan, successfully and can be used alone on a larger scale. Various modifications can now be used to determine other bilirubin/haem components. The

latter would finally enable the pediatricians to rely more on bilirubin estimation in faeces rather than serum for their management of children especially neonates (term and preterm newborns) with jaundice for the estimation of bilirubin. Type of disorder that can be investigated would be causes of unconjugated hyperbilirubinemia. Further research is indicated as also mentioned in the recent guidelines for the evaluation of cholestatic jaundice in infants, recommendations of the NASPGHAN (North American Society of Pediatric Gastroenterology and Nutrition).<sup>24,25</sup>

## Conclusions

The HPLC method described is effective for estimation of bilirubin in faeces of normal newborn and those with varying diagnosis. Our future plan is to do a study on a large sample of normal newborn from the nursery to serve as a cohort of controls. We may also look at ethnic variation in the excretion of bilirubin in future studies.

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