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DIAZEPAM AND ITS METABOLITES IN THE MOTHERS' AND NEWBORNS' HAIR AS A BIOMARKER OF PRENATAL EXPOSURE

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Pregnant women are exposed to benzodiazepines for therapeutic purposes during gestation. The goal of this study was to evaluate prenatal exposure to benzodiazepines. Time of exposure during course of pregnancy is a significant aspect of fetal exposure to drugs. Benzodiazepine concentration assay in hair of mothers and newborns exposed prenatally to these drugs was performed in the studies. Development, validation and evaluation of benzodiazepine determination method in mothers and their newborns enables assessment of health risks for the child and implementation of adequate therapeutic procedures. We used A LC-ESI-MS/MS method that allowed determination of diazepam (the main benzodiazepine used by pregnant women was diazepam) and its metabolites (nordazepam, oxazepam) in hair of mothers and newborns. LOQ 10 pg/mg of hair was used in the study. Results: concentration of nordazepam was higher than parent drug (diazepam) and higher in newborns' hair when compared to mothers'. The mean concentrations of diazepam in mothers' hair were 31.6±36.0 and 34.1±42.4 pg/mg in the second and third trimester of pregnancy respectively. The mean concentration of diazepam in newborns' hair was higher and reached levels of 53.3±36.5 pg/mg. The mean concentration of nordazepam in the mothers' hair corresponding to the second and third trimester was 52.9±48.1 and 89.9±122.8 pg/mg, respectively. Nordazepam in the newborns' hair was detected at the mean level of 108.1±144.2 pg/mg. It was concluded that diazepam and nordazepam are permanently incorporated into the hair structure. Presence of diazepam and its metabolites in newborn's hair confirms that these benzodiazepines permeate placental barrier. Segmental analysis of mothers' hair enabled the assessment of drug administration time. Diazepam and its metabolites determined in hair of newborns may serve as biomarkers of prenatal exposure to these drugs. The performed LC-MS/MS analysis was accurate enough to determine even low concentrations of benzodiazepines, at the level of few pg/mg of hair. Levels of diazepam detected in hair of newborns were higher than levels determined in mothers. This may confirm the fact, that fetus's ability to metabolize diazepam is scarce. Nordazepam was found in higher concentrations in hair of newborns than in hair of mothers, which may suggest that it is cumulated in child's organism. Other metabolites of diazepam - oxazepam and temazepam - were detected in very few cases, in low concentrations.

Key words: *benzodiazepines, diazepam, nordazepam, hair analysis, pregnancy, newborn, fetus, maternal plasma*

INTRODUCTION

Anxiety disorders connected with an active lifestyle prompt some pregnant women to seek for pharmacological intervention by their obstetricians. Benzodiazepines, especially diazepam, have been used by obstetrical providers as the major class of drugs for maternal therapy (1). The pharmacological intervention is justified because there is an association between high levels of anxiety during pregnancy and adverse neonatal outcomes. Premature births as well as low birth weight have been associated with untreated maternal anxiety during pregnancy (2). Benzodiazepines are one of the most commonly used anxiolytics in the world (3). They are prescribed to women of childbearing age and to pregnant women (4) in order to reduce stress and manage preeclampsia or eclampsia in the latter part of pregnancy. Most benzodiazepines diffuse across the placenta (5) leading to higher fetal levels when compared to levels detected among pregnant women. There have been concerns regarding the adverse effects of preservatives used in some preparations, which could displace bilirubin from albumin leading to hyperbiliruminemia among infants whose mothers were treated with diazepam. Moreover, the teratogenicity of benzodiazepines prescribed during pregnancy have been reviewed (6). When administered to a pregnant women at or near the time of delivery

benzodiazepines may cause "the floppy infant syndrome" (6, 7). Recovery from this condition can last for several hours after birth (7). Thus, if the use of benzodiazepines is necessary during pregnancy, the lowest effective dose of the short-active agent should be established.

There are several methods to determine the levels of benzodiazepines in hair (8). Most studies focus on the determination of benzodiazepine levels in adult hair (9); however, fewer studies have determined these drug levels in newborn hair. A method that would be suitable for analysis for both mother's and newborn's hair still awaits development and adoption into clinical or forensic practice. The goal of the study was to evaluate the prenatal exposure to benzodiazepines based on the determination of the benzodiazepines levels of maternal and newborn hair.

MATERIALS AND METHODS

Specimens

Blank dark hair samples for development and validation of a method were obtained from volunteers who did not take benzodiazepines, after their verbal consent. An informed consent for the procedure was obtained from 47 pregnant women at the Department of Perinatology and Gynecology of the Poznan University of Medical Sciences in Poznan, who had been prescribed benzodiazepines by their obstetrician for clinical anxiety. Samples from mothers and newborns were collected within 24 hours after delivery. Hair was clipped as close to the scalp as possible and stored at room temperature. The protocol for the study was approved by the Bioethics Commission University of Medical Sciences in Poznan, Poland (No. 387/08, April 3rd 2008). The study was conducted in compliance with the Helsinki Declaration (1975 with revision of 2000). Participation in the study was voluntary.

Hair samples (both for development of the methods and for experimental use) were decontaminated by subsequent washing with phosphate buffer pH 7.4 (10 ml) for 5 min, isopropanol (10 ml) for 5 min and methylene chloride (10 ml) for 5 min and dried in 40°C.

Mother's hair strands were cut into 3 cm segments, beginning with the end closest to the scalp and then more cut more distally which corresponds to the third, second and first trimester of gestation.

Newborns' hair samples were not segmented. The maternal segmented samples and entire sample of newborns' hair used for analysis weighed 10±2 mg. Hair were powdered in a ball mill (Pulverisette 23, Fritsch, Germany) for further analysis.

Chemicals

Acetonitrile, isopropanol, dichloromethane and formic acid were HPLC grade and purchased from Sigma-Aldrich, phosphoric buffer was delivered by Merck. Ingredients of phosphate buffer were purchased from (Polish Chemical Reagents, POCH, Poland). Diazepam, nordazepam, oxazepam, temazepam and diazepam-d5 in concentration of 1 mg/ml were purchased from LGC Standards (Dziekanow, Poland). Drug "standards" were prepared by dissolving an appropriate mass in methanol to provide a concentration which was subsequently diluted to required concentrations. An internal standard (IS) working solution of the diazepam-d5 was prepared (0.2 µg/ml in methanol), which was further diluted with methanol to yield appropriate concentrations to add to samples, calibrators and quality control samples. Working solutions were stored at 4°C up to one month.

Sample preparation

The diazepam-d5 20 µl (400 pg/mg) (IS) was added to approximately 10 mg of powdered hair (calibration, control and real samples). Next, incubation with 400 µl of phosphate buffer (pH 7.4) at 50°C was performed overnight. Subsequently, supernatants were transferred to 2 ml disposable screw top vials and extracted with 1.2 ml of diisopropyl ether (5 min). After centrifugation (4000 rpm for 15 min) the organic layer was collected and evaporated under nitrogen in temp. 40°C. The residue was reconstituted by adding 50 µl of the mixture of acetonitrile and water (1:1) with addition of 100 µl/100 ml of formic acid and 20 µl was injected into the liquid chromatography-electrospray ionization-tandem mass spectrometry (LC-ESI-MS/MS) system.

Chromatography and mass spectrometry

Chromatographic separation was performed with Agilent HP1200 series (Agilent Technologies USA). 20 µl of reconstituted samples were injected into Zorbax Eclipse XDB-C18 Rapid Resolution HT 50 × 4.6 mm column. The column was thermostated at 35°C. Chromatographic run was carried out with a gradient elution with 0.1% formic acid in water (A) and acetonitrile (B), at a flow rate of 0.5 mL/min.

A gradient was applied starting with 30% B, maintained by 3 min. and increased to 80% in the next 3 min than kept by 1 min. From 7 min to 8 min, B was decreased to 30% for stabilization of mobile phase. A total runtime was 13 min.

Detection was carried out by LC-ESI-MS/MS 6460 Triple Quad Mass Spectrometer. The instrument was operated in the positive ionization mode. Instrument sensitivity was optimised to obtain at least three transitions (MRM). Mass optimiser was applied for optimisation of fragmentor voltage because of its greatest impact on sensitivity and fragmentation. The objective was to find the optimum voltage which provided the strongest molecular ion and fragment ions.

The optimal parameters of mass spectrometer were: gas temperature 325°C and gas flow 10 L/min, nebulizer pressure 40 psi, sheath gas temperature 400°C and flow 10 l/min, the capillary voltage was 400 V. For this parameter optimisation, the concentration of each benzodiazepine in the standard mixture was 200 ng/ml.

LC-ESI-MS/MS assay validation

Calibration curves were obtained by preparing spiked blank, black hair (10 mg) containing final 0, 20, 50, 100, 200, 500 pg/mg of diazepam, nordazepam and oxazepam.

The assessed matrix effect was slightly enhanced for diazepam (127%±22) and oxazepam (110%±14) and showed no effect on both compounds. The assessment was performed with use of Matuszewski *et al*. method (10).

For the examination of intra-day and inter-day repeatability (replicate analysis performed per three days) and precision, control samples with concentration of 20, 100 and 500 pg/mg and for recovery 20 pg/mg, were prepared using blank hair spiked with the analytes and real hair samples.

The samples of hair fortified with diazepam, nordazepam and oxazepam were used for the development and validation of the quantification procedure for these compounds. In the conditions provided, a separation of the three quantified compounds occurred. In order to determine the specificity of the developed method six blank hair samples were checked for signals interfering with analytes. Under the chromatographic conditions there were no interference with analytes by any extractable endogenous material present in blank hair. Six-point standard curves were prepared by

fortifying blank hair with known quantities of studied analytes and deuterated diazepam and were linear in the studied range for all benzodiazepines. Correlation coefficients (r) were: 0.997 for nordazepam, 0.989 oxazepam, and 0.999 for diazepam. High correlation factors and repeatability confirm the suitability of the method for chromatographic analysis of compounds of interest in hair and even in small samples of newborn hair.

The intra-day precision was evaluated by analysis of five hair samples collected separately from volunteers who did not take diazepam. The mean values of relative standard deviation (R.S.D.) are presented in *Table 1*.

The limits of detection (LOD), which were the lowest concentrations of drugs at which the signal to noise ratio S/N was 3 on the similar level of 5 pg/mg for all studied benzodiazepines. The limit of quantification (LOQ), the lowest concentration on the calibration curves for all analytes was 10 pg/mg (*Table 1*). Recovery was in the range 87.5–111.6%.

Statistical analysis

The results are expressed as mean values and standard deviation. The Student's T test was used to assess statistical significance of differences between mean concentrations and ratios of studied compounds ($p<0.05$). Correlation between respective variables were indicated on the base of Pearson coefficient.

RESULTS

The clinical administered dose of diazepam ranged from 5 to 15 mg per day, and period of administration from 1 to 9 months. The mean concentration of diazepam in mothers' hair ranged from 30.8±11.6 pg/mg in the first trimester to 34.1±42.4 pg/mg in the third trimester. Although the mean concentration of diazepam in newborns' hair (53.3±36.5 pg/mg,) was higher than in the samples of mothers' hair in the third trimester the differences were not significant due to high variability of diazepam dosage and administration time in the group of participating women (*Table 2*).

The examples of diazepam and its metabolite's concentrations in hair of mother and newborn are shown in *Fig. 1*.

The ratio of concentration (pmol) of the main metabolite of diazepam-nordazepam in hair of women in the second and third trimester of gestation was approximately three times higher than diazepam, while in the hair of newborns it was five-fold higher than concentration of the parent drug and these differences were statistically significant (*Fig. 2*).

DISCUSSION

Currently searching for correlation between mother's health problems and medical condition of a child is increasingly perceived as an important prognostic factor for potential paediatric therapy. The research involve diseases of genetic descent and those caused by exposure to xenobiotics such as drugs or toxins (11, 12). Psychotropic drug use safety during pregnancy still raises a concern. Studies lack a coherent approach and uniform methodology, and thus results are often contradictory (13). Animal studies provide disturbing data concerning the benzodiazepine treatment in pregnancy, although they cannot be simply extrapolated to humans due to varying sample sizes and multiple drug/toxin exposures, which complicate the interpretation of the results (7). Exposure to high-dose benzodiazepines in utero has been associated with newborn withdrawal symptoms, but there is not enough data to confirm longterm effects of these drugs (14). Retrospective studies can be biased due to under-reporting of drug consumption by mothers (15). Further, case reports are influenced by limited sample sizes. Additionally, numerous factors affecting child development may further hinder the conclusions in follow-up studies. Prenatal exposure to legal or illicit drugs can be examined by determination of their metabolites in different materials collected before or after delivery (16). The simplest one is analysis of the newborns' first urine (reflects the exposure just before delivery), meconium, amniotic fluid or hair. Currently hair is the most frequently used material for evaluation of the prenatal exposure.

Table 1. Validation parameters of the LC/MS/MS method for benzodiazepines determination.

Parameter	**Concentration [pg/mg]**	**Compound**		
		Diazepam	Nordazepam	Oxazepam
Intra-day precision [RSD]	20	0.5	21.4	23
	100	5	12.1	19
	500	1.4	3.1	8
Recovery [%]	20	111.6	91	87
LOD [pg/mg]		5	5	5
LOQ [pg/mg]		10	10	10

Table 2. Mean values and standard deviation of dose, administration time of diazepam and diazepam and nordazepam concentration.

Period	Mean dose during period of treatment [mg/day]	Length of dosing [months]	Diazepam concentration [pg/mg]	n*	Nordazepam concentration [pg/mg]	n*
I trimester	7.6±3.6	2±0.9	30.8±15.3	6	64.5±61.5	4
II trimester		2±1	31.6±36.0	14	52.9±48.1	17
III trimester		2.5±0.7	34.1±42.4	26	89.9±122.8	28
Newborn			53.3±36.5	14	108.1±144.2	27

* – Number of results above LOD.

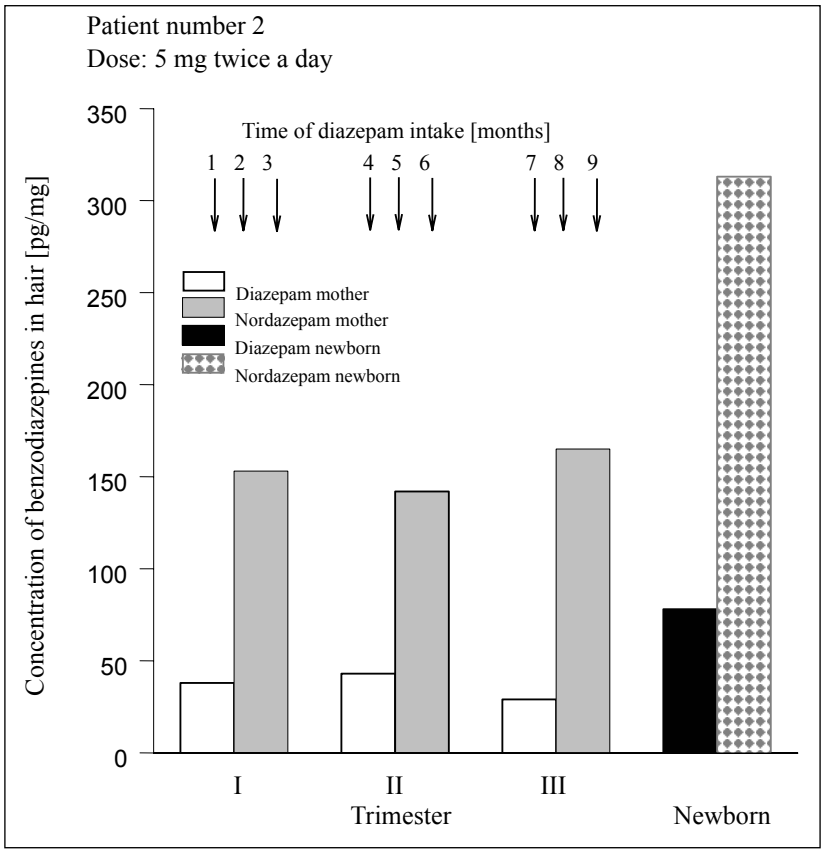

Fig. 1. Concentration of diazepam and nordazepam in hair of randomly selected patient (number 2) and her newborn.

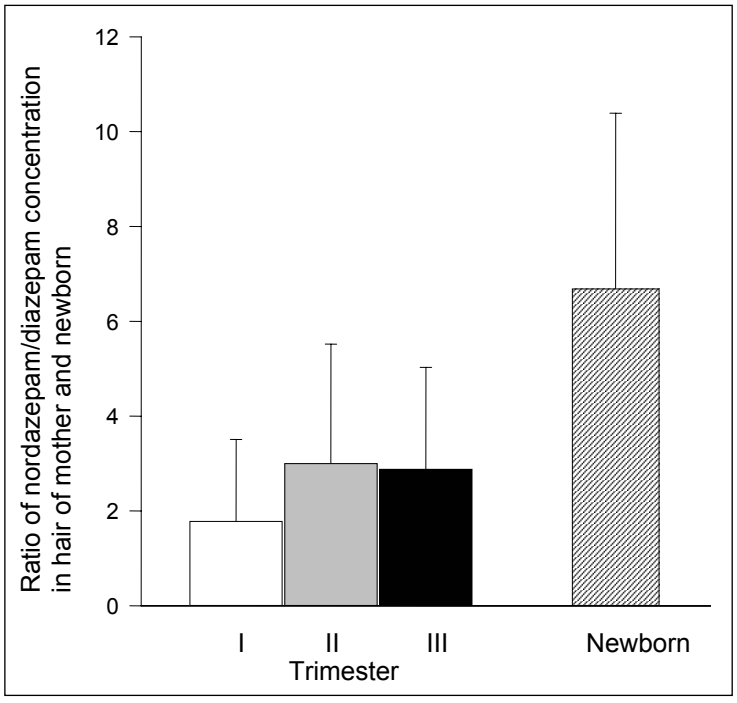

Fig. 2. Ratio of nordazepam/diazepam concentration (pmol) in hair of mother and newborn.

The concentration of diazepam in hair of women in our study was lower than in the study of McClean *et al*., however in their study the exact period of drug administration in relationship to analysis was not described (17). The lack of standardized techniques in the studies of drug's adverse effects is one of the obstacles in establishing fetal exposure. As herein reported, segmental analysis of mother's hair and her newborn may be an acceptable for detection of a biological marker of benzodiazepine use which remains sensitive weeks after the end of maternal consumption of these agents. This enables a cumulative documentation of long term exposure to these agents. Diagnostic ascertainment is feasible with the use of hair sampling when the maternal history is neither accurate nor available. This method has better recovery with similar precision

than method developed by Villain *et al*., although it has higher limit of quantification (13). Moreover, a further advantage of the current method was that only half as many samples were needed for drug levels determination, important in case of analyzing biological samples from newborn infants.

Only few studies reported the results of determination of benzodiazepines in neonatal hair (18, 19). We found that concentrations of diazepam in the hair of newborns were slightly higher (not significant) whereas concentrations of nordazepam were significantly higher than in mother's hair ($p<0.05$).

Contact of hair with amniotic fluid should be taken under consideration during interpretation of results of drugs determination in newborns' hair. The most probable time for contamination of hair by drugs contained in amniotic fluid is the third trimester. At that time fetal urine and lung fluid secretion become the two primary sources of amniotic fluid. Analysis of amniotic fluid for detection of drugs is still a largely unexplored option. Major studies have been focused on prenatal exposure to cocaine, in which both parent drugs and metabolites have been identified with concentrations in the range of nanograms per milliliter of fluid (20). According to the authors' best knowledge there is no data about concentration of benzodiazepines in amniotic fluid, what makes evaluation of its influence on concentration in hair impossible.

Studies focused on fetal exposure to benzodiazepines often lack information on duration and indication for use of benzodiazepines, medical/obstetric histories and family history, in addition to the fact that drug use histories from mothers may be unreliable. These all facts make assessment of risk associated with benzodiazepine use difficult (13, 21). Thus the analysis of mother's and newborn's hair present a very attractive method for determination of the drug levels. Further, these data combined with prospective analysis of the clinical outcomes including infant follow-up, may permit a better understanding of the longer term effects on infant development.

There is difference in the benzodiazepines profile in progression of pregnancy. This difference depends on greater placental transfer of benzodiazepines in late pregnancy when compared to early stages. This fact is related to an increased size of the placenta (up to 12 m^2 at the end of pregnancy (22)) and its lipid content. Additionally, the loss of placental cytotrophoblasts (23) and enhanced uterine circulation (approximately 10 to 20% of the maternal cardiac output in late pregnancy (24)) facilitate the transport of diazepam across the placenta. Diazepam crosses the placenta *via* both trans- and extracellular pathways at maximal or perfusion-limited rate leading to higher levels of the lipophilic drug in the fetus than in mother, until the concentrations steady-state is reached (25). Kanto *et al*. presented several studies which showed that in both early and late stages of pregnancy administration of diazepam (single and repeated dosing) results in elevated levels of diazepam and nordazepam in the fetus and, subsequently, newborn. Slow accumulation of diazepam in the fetus and its presence in fetal tissues even after the end of mother's therapy (26) might be caused by higher fetal plasma protein binding levels. Moreover, maternal protein binding capacity is lower than in the age-matched non-pregnant women (13). Because of these properties the total concentration of diazepam may be two-fold higher in the cord plasma during delivery than in the maternal plasma (fetomaternal ratio 1.2 to 2) during early pregnancy (26) and at term (5). Thus, the fetal and neonatal health condition may be endangered due to diazepam single high intravenous dose or repeated oral dosing administered to the mother (21, 27) before labor and delivery.

Neonatologists are concerned about the third trimester fetal exposure to benzodiazepines and their use during labor because of effects on the fetus and newborn. It has been reported that such fetal exposure carries the risk of "the floppy infant syndrome" or symptoms of withdrawal. These symptoms vary from mild sedation, hypotonia, and reluctance to suck, to apnoeic spells, cyanosis, and impaired metabolic responses to cold stress (28). Pharmacokinetic profile and placental transfer of the benzodiazepines and their deposition in the neonate expressly explain long-lasting occurrence of these symptoms - even several months after exposure (29).

Clinical use of pharmacologic agents during the prenatal period should always be preceded by safety testing. Verification of a drug involves preclinical studies including appropriate toxicological studies as well as appropriately designed phase III clinical trials. One of the most important factors characterizing the drug's features is its pharmacokinetics which is measurably altered during pregnancy. As clinicians evaluate the best means of monitoring the safety and efficacy of therapy (6), the attempt to develop a suitable method of neonate's and mother's hair analysis for assessment of benzodiazepine exposure level is needed.

We have developed a non-invasive method that can be applied for evaluation of prenatal exposure to benzodiazepines in newborns. In our study of 47 mother-newborn pairs, diazepam and especially the main metabolite of diazepam (nordazepam) are present in hair of mother and newborn after anxiety treatment with benzodiazepines during pregnancy. To expand these preliminary results to a broader population, a larger experimental group would be needed, but nonetheless we provide evidence of a sensitive and specific method for measurement of diazepam and its major metabolite in the hair of mothers and newborns. This method has both clinical relevance in neonatal care of children of mothers with a history of diazepam use during pregnancy and for forensic purposes. Although psychotropic medication is potentially toxic to the developing fetus the adverse effects were not well investigated likely due to the clinical, ethical, and legal aspects of fetal involvement in research (30). In view of these facts and based on the obtained results the authors suggest that the analysis of the mothers' and/or newborn's hair might be sufficient enough for the fetal benzodiazepine exposure evaluation.

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