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Binding of Thiopental in Neonatal Serum

Harry G. G. Kingston, M.B., B.Ch., F.F.A.R.C.S., Angela Kendrick, M.D.,† Karen M. Sommer, B.S.,‡ George D. Olsen, M.D.,§ Hall Downes, M.D., Ph.D.§*

Protein binding of thiopental was studied in 21 samples of neonatal serum (from placental blood) and compared with protein binding in ten healthy adult volunteers. These infants ranged between 32 and 43 weeks of gestational age (mean, 37.7 weeks) and the adult age range was from 27 to 54 years (mean, 35.4 years). Because the unbound fraction of the drug is responsible for its pharmacologic effect, a marked difference in the protein binding between neonates and adults may be relevant to the clinician. Blood obtained from freshly delivered placentas or from adult volunteers was allowed to clot and the serum separated from the sample. A portion of the serum was sent for protein and bilirubin analysis and the remainder retained for study. This latter serum was combined with four concentrations of thiopental. These specimens were then ultrafiltered and the amount of thiopental in the ultrafiltrate (unbound) compared with the prefiltered amount (total), as measured by reverse-phase high-performance liquid chromatography. The binding studies were repeated at *p*H 7.2, 7.4, and 7.6 in both the adult and neonatal serum. Total protein and albumin are significantly less in neonatal serum, whereas bilirubin (total and direct) is significantly higher in neonatal serum than in adult serum ($P < 0.01$). Neonatal serum was associated with significantly more unbound thiopental than adult serum at all levels of *p*H studied ($P < 0.005$). Increasing the *p*H resulted in less free drug in both groups, but this reached statistical significance only in the adult group ($P < 0.025$). Drug concentration had no effect on binding in the range examined. This increased unbound thiopental (almost twice as much as in adults) may result in increased sensitivity to thiopental in the neonatal age group. (Key words: Age: neonate. Anesthesia: pediatric. Anesthetics, intravenous: thiopental. Pharmacokinetics: protein binding.)

THERE ARE age-related differences in body composition between adults and infants that influence the activity, metabolism, and distribution of drugs. There are also quantitative and qualitative differences in neonatal and adult serum proteins that result in less binding of drugs by neonatal plasma in comparison with adults.[1,2] These differences do not persist beyond a few months of age as evidenced by the studies of Sorbo *et al.*, who found no difference in the protein binding of thiopental of children between 5 months and 13 yr compared with a group of adult volunteers.[3] No data are available that specifically describe the binding of thiopental in children younger than 5 months of age.

Protein binding is influenced by many factors including changes in serum *p*H. Because neonates may be acidotic as a result of the stresses of the birth, this could produce a difference in the protein binding of drugs; consequently, the amount of resulting unbound drug may be of clinical significance.

The purpose of this study was to measure the protein binding of thiopental in adult and neonatal serum at four concentrations of the drug representative of plasma concentrations both within and outside of the expected clinical range. Thiopental binding was also measured at each of these concentrations at *p*H 7.2, 7.4, and 7.6 to establish whether there were important changes in protein binding of thiopental over a *p*H range seen in clinical practice.

Methods

For neonatal studies blood was obtained from the umbilical veins of 21 placentas at the time of vaginal delivery. These infants were from 32 to 43 weeks of gestational age (mean, 37.7 weeks). Blood from placentas of mothers who had received a barbiturate was excluded from the study but blood from patients who required epidural anesthesia using bupivacaine was used. Because the volume of blood obtained from the umbilical vein was limited and variable, the samples were divided such that at any one drug concentration, all three *p*H values were usually studied on serum from the same placenta. In addition, at any given drug concentration and *p*H, the n was 10. Blood was also obtained from ten healthy adult volunteers (nine males and one female whose ages ranged from 27 to 54 yr; mean, 35.4 yr). All blood samples were collected in heparin-free vacutainer tubes. The blood was allowed to clot and a portion of the serum was sent for measurement of total protein, albumin, and bilirubin (total and direct). The remainder of the sample was centrifuged for 20 min, and the serum was retained for binding studies. When the studies were not undertaken on the day of obtaining the placental blood, the serum was separated as described, frozen at −15° C and the analysis performed later. Kurz and Trunk[4] had previously demonstrated that freezing does not change the binding properties of plasma.

Four concentrations of thiopental were made in a 5 mM solution of Hepes buffer (N-2-hydroxyethylpiperazine-N-2-ethanesulphonic acid, Calbiochem). These were designed so that when 200 μl of each concentration was added to a 1.8-ml sample of serum, a concentration of thiopental was achieved that encompassed those predicted

* Professor, Department of Anesthesiology.
† Assistant Professor, Department of Anesthesiology.
‡ Research Assistant, Department of Pharmacology.
§ Professor, Department of Pharmacology.
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Address reprint requests to Dr. Kingston: Department of Anesthesiology, Oregon Health Sciences University, 3181 SW Sam Jackson Park Road, Portland, Oregon 97201-3098.

to occur when 4 mg/kg thiopental is administered to a patient to induce anesthesia. These concentrations ranged from 7.9 to 79.2 μg/ml (3×10^{-5} to 3×10^{-4} M).

To study the possible effect of *p*H on protein binding, each of the four drug concentrations was made in triplicate (A, B, and C), and the *p*H was adjusted to 7.2, 7.4, and 7.6, respectively, using a calibrated *p*H meter. This was done by adding 0.01 to 0.03 ml of 1N NaOH or 1N HCl to 2.0 ml of the sample (using a 1-ml syringe and a 22-G needle) until the requisite *p*H was achieved.

The protein binding of serum was determined by using an Amicon MPS-1 micropartition ultrafiltration system (Amicon Corp, Lexington, Massachusetts) as described by Burch and Stanski.[5] By this method 1 ml of serum with thiopental is added to a filtration chamber in which the serum is separated from a collection cup by a YB-30 membrane. Centrifugation of the chamber for 30 min results in the accumulation of a maximal amount of protein-free filtrate (approximately 200–400 μl) in the collecting cup. The membrane used does not bind thiopental.[5] Because the membrane is impermeable to protein, the thiopental present in the ultrafiltrate is in the unbound form.

The addition of acid or base to the prefiltrate can result in a dilutional error, and because inaccuracies can occur when small volumes of fluid are pipetted, we analyzed the concentration of thiopental in both the prefiltrate (after adjusting the *p*H) and the ultrafiltrate. The protein binding of the sample was determined by comparing the drug concentration in the ultrafiltrate with the concentration in the prefiltered sample. The percentage of free drug could therefore be calculated. All drug additions, extractions, and measurements were conducted at room temperature.

Samples were analyzed with reverse-phase high-performance liquid chromatography (HPLC) with ultraviolet detection using the following modifications of the method described previously by Avram and Krejcie.[6] One hundred microliters of 0.01 M sodium phosphate buffer (*p*H 5.6) was injected onto a Chromprep PRP-1 80-μl cartridge (Hamilton Co, Reno, Nevada) preconditioned with methanol and water. Eighty microliters of diluted or undiluted plasma or ultrafiltrate containing 1.92 μg of secobarbital as an internal standard was injected onto the cartridge. After washing the cartridge with 300 μl of water, the drugs were eluted with 300 μl of methanol. The eluate was dried under N_2 with low heat and subsequently reconstituted in 300 μl of mobile phase. One hundred microliters were injected into the HPLC system and separated on a 100 × 3.2 mm, 3-μm particle, reverse-phase cartridge column (Velosep, Applied Biosystems Inc, Santa Clara, California) at a mobile phase flow rate of 0.75 ml/min. Using these modifications, the lower limit of detection and quantitation was 2.5 ng in an injection volume of 100 μl. Thiopental recovery from extraction was 80% with a coefficient of variation of 2.2% within assay and 4.6% between assays, determined at the midpoint of the standard curve. Considering the injection volume of 100 μl and the dilution inherent in the extraction procedure, it is possible to detect and quantitate values as low as 117 ng/ml thiopental in 80-μl samples. Standard curves were obtained by analysis of the plasma with 117–15,000 ng/ml of thiopental. The ratios of the areas under the peak of thiopental to secobarbital *versus* the thiopental concentrations gave standard curves determined by linear regression analysis with an average r value of 0.999.

The effect of age, drug concentration, and *p*H and the interaction of these on protein binding were analyzed by a three-way analysis of variance (ANOVA) with one repeated measurement (*p*H) (STATPAK™, North-West Analytical Inc, Portland, Oregon). The significance of increasing *p*H was further analyzed by least square linear regression analysis for both the adult and neonatal groups. Differences in biochemical measurements for the adult and neonatal groups were analyzed by Student's t test for unpaired data. A result was considered to be statistically significant if $P < 0.05$.

Results

Neonates had significantly higher total and direct bilirubin concentrations and lower total protein and albumin concentrations than adults. These differences are summarized in table 1.

There was a significant age ($P < 0.005$) and *p*H ($P < 0.025$) effect but no effect of drug concentration on the protein binding of thiopental. No interactions among age, *p*H, and drug concentration were detected by three-way analysis of variance. The *p*H effect was further analyzed and regression analysis showed that the protein binding of thiopental increased significantly with increasing *p*H in the adult group ($y = 5.25x + 45.58$, $P < 0.025$, $r = 0.234$) but not in the neonatal group (table 2). This *p*H effect was small and accounted for only a small fraction of the variation in binding and is therefore not of clinical significance.

TABLE 1. Adult and Neonatal Values of Bilirubin (total and direct), Total Protein, and Serum Albumin

	Adult (n = 10)	Neonatal (n = 10)
Total bilirubin (mg/dl)	0.56 ± 0.07*	1.77 ± 0.20
Direct bilirubin (mg/dl)	0.10 ± 0.02*	0.19 ± 0.03
Total protein (g/dl)	7.09 ± 0.13*	6.06 ± 0.28
Albumin (g/dl)	4.58 ± 0.12*	3.90 ± 0.18

Values are mean ± SEM.
* $P < 0.01$, adult *versus* neonatal.

TABLE 2. Mean Values of Protein Binding in Adult and Neonatal Groups at Each of Four Concentrations of Thiopental

Thiopental Concentration	*p*H 7.2	*p*H 7.4	*p*H 7.6
Adult group*			
3×10^{-5} M	82.6 ± 2.1	83.5 ± 1.4	84.4 ± 2.0
1×10^{-4} M	83.8 ± 0.9	86.0 ± 0.9	87.8 ± 0.5
2×10^{-4} M	83.7 ± 0.8	83.9 ± 0.7	84.7 ± 1.0
3×10^{-4} M	82.9 ± 1.2	84.3 ± 1.3	85.0 ± 1.0
Neonatal group*			
3×10^{-5} M	72.4 ± 3.5	76.4 ± 3.1	75.0 ± 2.1
1×10^{-4} M	72.8 ± 2.7	74.5 ± 2.6	76.0 ± 2.1
2×10^{-4} M	68.4 ± 4.2	69.9 ± 3.6	74.4 ± 3.0
3×10^{-4} M	71.3 ± 3.2	71.9 ± 2.8	73.2 ± 3.0

Values are the percentage of mean protein binding ± SEM.
* $P < 0.005$ adult *versus* neonatal groups.

Discussion

The amount of free drug in plasma is related to the protein binding, which in turn depends upon the concentration of protein, the number of binding sites, and the affinity constant of the drug for the protein.[7] Thiopental is known to be bound between 85% to 95% in adults.[8,9] Information about the specific binding of thiopental by neonatal serum is scanty, but because other drugs including phenobarbital appear to be less bound than in an adult, it is assumed that thiopental follows this pattern. Albumin, α-1 acid glycoprotein (AAG), and the lipoprotein fraction are the three main protein types associated with the binding of drugs. This is further modified by age,[10,11] disease states,[12–14] and competitive and noncompetitive binding by other pharmacologic substances or plasma constituents. As an acidic compound, thiopental is bound mainly to albumin.[15]

There are factors that specifically influence protein binding in the neonatal age group. These include the presence of maternal steroids and especially the presence of unconjugated bilirubin[2] and free fatty acids,[16] all of which reduce the number of potential binding sites. The neonate has decreased total plasma proteins and albumin compared with the adult.[1] They also have persistent fetal albumin, which may have different binding characteristics from those of more mature protein and disappears within the first months of life.[17] The concentration of serum albumin in children reaches that in adults by infancy, whereas total protein remains at a lower concentration than in adults largely because of reduced globulin levels, which increases later in childhood.[7] Our findings are in agreement with this pattern and demonstrate these differences in protein and bilirubin between adults and neonates. We did not measure nonesterified fatty acids (NEFA), but other investigators have shown these to be elevated in neonates, and, like biliburin, reach adult levels with the onset of infancy.[7] It would be tempting to explain the increased free drug on the basis of the lower plasma concentration of neonatal protein (specifically albumin). In a comparison of protein binding of a variety of compounds, Kurz *et al.*[1] showed that the diminished protein binding of neonatal plasma persisted even when the concentration of plasma protein was adjusted up to adult levels using ultrafiltration.

Therefore, the differences in binding demonstrated in this study are more probably due to a structural difference between adult and fetal albumin or the presence of increased concentrations of substances, such as bilirubin, which are known to decrease the binding capacity of serum protein.[2]

It is also possible to increase the unbound fraction of a drug by saturating the binding sites with increased serum concentrations of that drug. Morgan *et al.*,[9] in a study of adult patients, have shown that if the serum concentration of thiopental is raised above 150 μg/ml, saturation of the albumin binding sites occurs. Because the highest serum concentration in this study was 79.2 μg/ml, a decrease in binding as a function of increased thiopental was not observed. Because of the lack of concentration-dependent binding in the range studied, Scatchard analysis was not done; therefore, the binding affinity and number of binding sites were not determined.

Birth may be associated with metabolic acidosis in the infant and the serum *p*H of a neonate may be decreased for some days. One might expect this to result in larger amounts of free thiopental if a neonate were anesthetized within days of birth. A *p*H effect was demonstrated in the sera of adult patients in which the differences at the three levels of *p*H studied reached statistical significance. This was not seen in the samples of neonatal blood, presumably because the scatter of the data in the pediatric group was greater than that of the adult group. Although the *p*H effect may be of statistical interest, the authors doubt whether it is of practical significance because the small changes demonstrated would be of little relevance during anesthesia.

Neonatal serum binds thiopental significantly less than adult serum. Many factors may cause this in neonates, but it most likely results from the competitive binding of the available receptor sites by other serum substances, such as bilirubin, which is known to be elevated in neonatal blood. Westrin *et al.*,[18] in a study of the thiopental requirements for induction of anesthesia in neonates compared with infants of 1–6 months of age, found that a smaller dose of thiopental is necessary in the neonatal group. Although the absolute difference in protein binding between adults and neonates is small, the resulting free drug fraction in neonates is 1.5–2 times as great as that in adults. Thus, in adults there was 15–17% free thiopental whereas 22–30% was unbound in the neonatal

group. This result and differences in blood–brain barrier permeability and CNS myelination may contribute to an increased sensitivity of a neonate to thiopental in the early weeks of life.

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