Veterinary
Science & Technology

Clemmons et al., J Veterinar Sci Technolo 2013, 4:5
http://dx.doi.org/10.4172/2157-7579.1000143

Research Article Open Access

Free-to-Bound Serum Phenobarbital Levels in Dogs

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Abstract

Monitoring phenobarbital levels in dogs has not traditionally taken free-to-bound serum phenobarbital levels into consideration. This study was designed to evaluate free-to-bound phenobarbital in healthy research dogs during the initiation of therapy with phenobarbital. These findings were compared with the levels obtained during routine therapeutic monitoring of phenobarbital in epileptic dogs. This appears to be the first descriptive study which has looked at free-to-bound phenobarbital in clinical canine patients. During the first week of therapy in healthy canine subjects, the free-to-bound ratio was 0.7 due to the high degree of protein-binding of serum phenobarbital. This was in contrast to the level of protein-binding in epileptic patients receiving phenobarbital chronically, with a free-to-bound ratio of 3.0 ± 2.7. Moreover, during the initiation of therapy with phenobarbital, some of the bound fraction could be displaced with sulfadimethoxine indicating that free-to-bound phenobarbital may have significance during this period. On the other hand, chronic therapy in epileptic dogs leads to reduction in the bound fraction for serum phenobarbital. This leads to a strong, direct correlation of total and free phenobarbital in these patients. This relationship is consistent over a wide range of serum concentrations and does not appear to be affected by the age of the patient, the length of chronic therapy or the presence of other anticonvulsant medications. There was an inverse relationship with total plasma proteins and free phenobarbital concentrations, but it was not statistically significant. Routine monitoring of free-to-bound phenobarbital may not be warranted except under specific conditions.

Keywords: Free-to-bound phenobarbital; Dogs; Epilepsy

Introduction

Phenobarbital is one of the first-line anticonvulsants used in the treatment of canine seizures [1]. It is generally safe, effective and well tolerated. At the same time, some patients do develop transient sedation and ataxia and many develop polydypsia, polyphasia and polyuria. Despite adequate total phenobarbital levels, some patients still do not reach satisfactory seizure control [1]. This may be secondary to changes in free-to-bound phenobarbital concentrations in the serum, since only the free portion of serum phenobarbital crosses the blood brain barrier to have anticonvulsant effects [2]. Studies on free-to-bound phenobarbital concentrations in serum are limited in dogs and usually reported in samples where phenobarbital is added directly in vitro into serum [3-5]. While this may give some information about protein binding, in vitro mixing of phenobarbital with serum may not be the same as occurs within the body. In human patients on phenobarbital, most of the available data suggests that there is approximately a 1:1 ratio of free and bound phenobarbital so that 50% is free and 50% is bound [6]. The bound fraction appears to be mostly bound to serum albumin and is not displaceable. In that situation, knowing the total level of phenobarbital is adequate, since the level of free phenobarbital that is available to the CNS can be predicted from the total level. The purposes of this study is to assess the levels of phenobarbital in research dogs given oral phenobarbital and in clinical patients during routine phenobarbital monitoring to evaluate whether free to bound ratios of phenobarbital are similar to human beings or unique to dogs.

Materials and Methods

Animals

The project was broken into 2 parts for this research. The first study group was performed on phenobarbital, naïve dogs housed in the UF Animal Resources facilities according to USDA and AAALAC guidelines in an IACUC approved protocol. These dogs were healthy, mix breeds between 2-5 years of age and ranged for 20-30 kg in weight. They were fed Purina Dog Chow at 1200 hours (the food was removed at 1600 hours if not consumed). They were provided ad lib water and exercised and socialized daily at 1400 hours. Phenobarbital was administered orally at 4 mg/kg every 12 hours at 800 and 2000 hours (EST). Blood samples will be collected at 800 and 2000 hours (at time of medication [trough dose]) and 1000 and 2200 hours (peak for dose) daily during the study. On the eighth day, each patient also received at 1000 hours 15 mg/kg of sulfadimethoxine IV to see if there is an effect on protein binding and the available free phenobarbital level one hour later (1100 hours).

The second study group was canine epileptic patients where phenobarbital was used as part of the patient's anticonvulsant regime. During routine monitoring of the patient's serum phenobarbital levels, a portion of the sample was used to determine the free phenobarbital level (as well as the total level). Each sample (if a serum chemistry profile was not clinically performed) had a serum total protein concentration determined.

Sample Analysis

Serum samples were obtained via cephalic or jugular venipuncture into non-separator serum tubes and centrifuged, allowed to clot and then centrifuge to separate the serum. Phenobarbital levels were either measured immediately or the serum was frozen at -20°C until analyzed.

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Received July 08, 2013; **Accepted** September 06, 2013; **Published** September 09, 2013

Citation: Clemmons RM, Schubert TA, Peters RK, Conrad BL (2013) Free-to-Bound Serum Phenobarbital Levels in Dogs. J Veterinar Sci Technol 4: 143. doi:10.4172/2157-7579.1000143

Phenobarbital levels were measured using a chemiluminescent enzyme immunoassay according to the manufacturer's instructions (Immulite 1000 Phenobarbital, Siemens Healthcare Diagnostics, Malvern, PA). In addition to the total serum level, 500 µl of serum will be centrifuges at 12,000 g for 10 minutes through a centrifugal filter device with a 30,000 MW cutoff, providing a protein-free, serum filtrate (Amicon Ultra-0.5 30K device, Millipore Corp., Billerica, MA) [6]. For those samples without a concurrent serum chemistry profile, the serum total protein concentration was performed using a Coomassie Blue protein assay (Bio-Rad Laboratories, Hercules, CA).

Data Analysis

Total and free phenobarbital concentrations were used to determine bound phenobarbital levels, the free-to-bound phenobarbital ratio, and the percentage of free or bound phenobarbital in serum. The data from the first group were plotted to see the effects time on peak and trough levels and if the free-to-bound phenobarbital levels varied with time or concentration. Data will also be analyzed using a statistical program (SPSS 14.0 for windows, SPSS Inc. Chicago, IL) to compare free-to-bound concentrations at each time point among the dogs for the accumulative data. For the clinical patients, mean data from epileptic patients will be analyzed statistically to calculate the distribution of total, free and bound phenobarbital levels. The accumulative level of the research dogs and the mean level for the clinical patients were compared, as well. The level of significance was set at $p \leq 0.05$ for comparison of means. For the sulfadimethoxine trial, the assessment was performed using the student's paired *t*-test. Other measurements were done with ANOVA or through Pearson's correlation coefficient with 2-tailed tests of significance.

Results

Phenobarbital levels increased rapidly during the first few days after beginning the drug in healthy dogs reaching and maintaining therapeutic concentrations (≥ 15 µg/ml) within 36 hours (Figure 1). After the first few days, there are a decline in the serum levels concurrent with plasma dilution (data not shown) secondary to polydipsia and polyuria which was noted in the dogs. In addition, on day 5 through day 7, the dogs were noted to be more sedate than normal during their exercise periods. Both of these effects were transient and required no special attention. The concentration of serum free phenobarbital paralleled the changes in total levels providing a fairly consistent free-to-bound phenobarbital ratio of 0.7 during the study. This led to a relative percentage of free phenobarbitalof 38.4 ± 2.2 and a relative percentage of bound phenobarbital of 58.2 ± 2.2 (Table 1). The peak and trough values reach a consistent state during the last 36 hours of the study period and varied by approximately 4 µg/ml (28.0 ± 0.4 minus 24.1 ± 0.8 µg/ml, respectively).

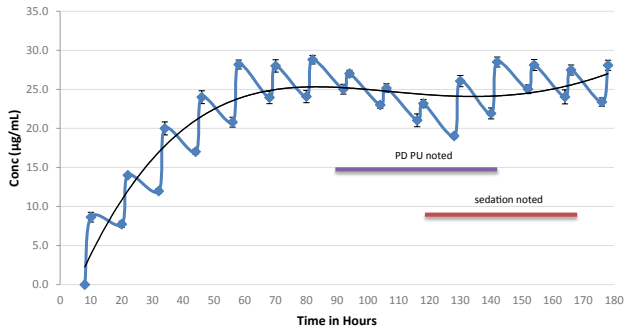

Figure 1: Peak and trough serum Phenobarbital levels in normal dogs (n=5) given oral Phenobarbital at 4 mg/kg every 12 hours. Levels are expressed in µg/ml for the total Phenobarbital concentrations taken before each medication (trough) period and 2 hours after medication (peak). Bars represent SD of the mean value. There was a dip in the total Phenobarbital levels that occurred around the 4-6 day when patients were observed to exhibit increased thirst (PD) and urination (PU). In addition, a transient sedation was noted around day 5 which continued through day 7.

Time		total	free	bound	f/b ratio	% free	% bound
8	mean	0.0	0.0	0.0	0.0	0.0	0.0
	SD	0.0	0.0	0.0	0.0	0.0	0.0
10	mean	8.6	3.4	5.2	0.7	39.5	60.5
	SD	0.6	0.2	0.5	0.1	2.2	2.2
20	mean	7.7	3.2	4.6	0.7	41.0	59.0
	SD	0.4	0.3	0.2	0.0	1.6	1.6
22	mean	14.0	5.6	8.4	0.7	39.9	60.1
	SD	0.2	0.1	0.2	0.0	1.1	1.1
32	mean	12.0	4.3	7.7	0.6	35.9	64.1
	SD	0.3	1.3	1.4	0.2	11.3	11.3
34	mean	20.0	7.3	12.7	0.6	36.7	63.3
	SD	0.8	1.2	1.4	0.1	6.0	6.0
44	mean	17.0	6.8	10.3	0.7	39.7	60.3
	SD	0.3	0.2	0.3	0.0	1.2	1.2
46	mean	24.0	9.6	14.4	0.7	40.0	60.0
	SD	0.8	0.5	0.5	0.0	1.2	1.2
56	mean	20.8	8.2	12.6	0.7	39.6	60.4
	SD	0.6	0.3	0.4	0.0	0.4	0.4
58	mean	28.2	11.2	17.0	0.7	39.8	60.2
	SD	0.6	0.2	0.4	0.0	0.5	0.5
68	mean	24.0	9.6	14.4	0.7	40.0	60.0
	SD	0.8	0.4	0.5	0.0	0.5	0.5
70	mean	28.0	11.1	16.9	0.7	39.8	60.2
	SD	0.8	0.4	0.4	0.0	0.3	0.3
80	mean	24.1	9.7	14.4	0.7	40.3	59.7
	SD	0.8	0.4	0.5	0.0	0.4	0.4
82	mean	28.8	11.4	17.4	0.7	39.7	60.3
	SD	0.5	0.3	0.4	0.0	0.7	0.7
92	mean	25.0	10.0	15.0	0.7	40.0	60.0
	SD	0.6	0.3	0.4	0.0	0.4	0.4
94	mean	27.0	10.7	16.4	0.7	39.4	60.6
	SD	0.4	0.4	0.2	0.0	1.1	1.1
104	mean	23.0	9.2	13.8	0.7	40.0	60.0
	SD	0.4	0.1	0.3	0.0	0.3	0.3
106	mean	25.2	10.0	15.1	0.7	39.9	60.1
	SD	0.6	0.3	0.3	0.0	0.3	0.3
116	mean	21.0	8.4	12.6	0.7	40.0	60.0
	SD	0.8	0.4	0.5	0.0	0.9	0.9
118	mean	23.2	9.3	13.8	0.7	40.3	59.7
	SD	0.5	0.3	0.5	0.0	1.2	1.2
128	mean	19.0	7.7	11.4	0.7	40.3	59.7
	SD	0.3	0.2	0.4	0.0	1.3	1.3
130	mean	26.1	10.3	15.7	0.7	39.6	60.4
	SD	0.7	0.2	0.6	0.0	0.9	0.9
140	mean	21.9	8.9	13.0	0.7	40.5	59.5
	SD	0.7	0.3	0.4	0.0	0.5	0.5
142	mean	28.5	11.3	17.2	0.7	39.7	60.3
	SD	0.6	0.2	0.5	0.0	0.6	0.6

152	mean	25.0	10.1	14.9	0.7	40.3	59.7
	SD	0.6	0.4	0.2	0.0	0.6	0.6
154	mean	28.1	11.2	16.9	0.7	39.9	60.1
	SD	0.7	0.3	0.5	0.0	0.6	0.6
164	mean	24.0	9.7	14.3	0.7	40.3	59.7
	SD	0.9	0.4	0.6	0.0	0.9	0.9
166	mean	27.5	10.9	16.6	0.7	39.6	60.4
	SD	0.6	0.3	0.4	0.0	0.5	0.5
176	mean	23.4	9.4	14.0	0.7	40.2	59.8
	SD	0.5	0.2	0.3	0.0	0.3	0.3
178	mean	28.1	11.2	16.9	0.7	39.8	60.2
	SD	0.6	0.3	0.5	0.0	0.6	0.6

Table 1: Peak and trough serum Phenobarbital levels in normal dogs given oral phenobarbital every 12 hours for 1 week. Levels are expressed in µg/ml for total, free and protein-bound phenobarbital serum levels. Samples were taken before medication and 2 hours following administration of 4 mg/kg dosage. Data are as mean and standard deviation (n=5).

Time		total	free	bound	f/b ratio	% free	% bound
Pre	mean	28.1	11.2	16.9	0.7	39.8	60.2
	SD	0.6	0.3	0.5	0.0	0.6	0.6
Post Sulfa	mean	28.0	14.0*	14.0*	1.0*	49.9*	50.1*
	SD	0.5	0.3	0.3	0.0	0.3	0.3

Table 2: Phenobarbital levels in normal dogs on oral phenobarbital before and 1 hour after IV sulfadimethozine administration (15 mg/kg). Levels are expressed in µg/ml for total, free and protein-bound phenobarbital serum levels. Samples were taken 2 and 3 hours following administration of 4 mg/kg of phenobarbital. Data are as mean and standard deviation (n=5). While the total phenobarbital levels are not statistically significant, all other measures were significantly different ($p<0.05$*, student's paired t-test).

The results for total phenobarbital are consistent with clinical data which suggests that a starting oral dose of 4 mg/kg every 12 hours will accomplish blood levels in the middle of the therapeutic range (mid- to upper 20's). Phenobarbital is highly protein bound in most species including dogs. Our findings in this part of the study are consistent with this, as the bound portion of the serum phenobarbital made up a majority of the total serum phenobarbital level. In fact, during this initial week of therapy, the bound portion of phenobarbital was in excess of that reported for other species. To investigate whether the bound fraction was permanently bound to plasma proteins or could be displaced by other medications known to displace protein-binding of drugs, sulfadimethoxine was given to the dogs at the end of the experiment and the free-to-bound phenobarbital levels were investigated (Table 2). There was a significant increase in the free fraction of serum phenobarbital with a concordant decrease in the bound fraction following sulfadimethoxine administration. This led to a free-to-bound ratio of 1 with a percentage of free phenobarbital of 49.9 ± 0.3 and bound phenobarbital of 50.1 ± 0.3. These changes were significantly different ($p \leq 0.05$) suggesting that around 10% of the bound phenobarbital in the dogs in the study were displaceable from their protein binding sites.

The second study group were samples collected from epileptic patients being treated with phenobarbital as part of their anticonvulsant therapy and as routine monitoring of their condition. These patients ranged from 0.5 to 14 years of age (Table 3). Four-fifths of the patients were male and one-fifth was female. One-fourth of the males were intact, whereas all the females were neutered. Three-quarters were purebred dogs (various breeds) and the remainder was mix-breeds. The duration of phenobarbital therapy was from 2 weeks (initial therapeutic monitoring period) up to 120 months. Many of the patients were on additional therapies, but there was no consistent additional medication or effect noted (data not shown).

Total phenobarbital levels in serum of epileptic patients varied greatly in patients examined and ranged from sub-clinical levels (10.8 µg/ml) to probably toxic levels (79.4 µg/ml). The latter levels were in one patient treated with a high loading dose of phenobarbital due to presentation to the UF Emergency and Critical Care for status epilepticus (part of the patient's typical pattern). The average age of epileptic patients was 6.9 ± 3.4 years and the average duration of therapy with phenobarbital was 24.6 ± 32.4 months. There was no significant change in free-to-bound phenobarbital parameters based upon sex, age, concurrent medications or length of medication. There was an inverse relationship between serum total protein and free phenobarbital concentrations, but this was not significant ($p=0.11$). On the other hand, the free phenobarbital was directly proportional to the total phenobarbital ($r=0.976$, Figure 2).

The free-to-bound ratio in epileptic dogs was 3.0 ± 2.7 with a percent free phenobarbital of 68.9 ± 11.3 and percent bound of 31.1 ± 11.3. While there was more variation in absolute levels in epileptic patients compared with the research dogs, this might be expected from their varied lifestyle and dietary differences [7-9]. At the same time, the free-to-bound parameters were significantly different from those compared to the research dogs (analysis not shown). This seemed to be due to decreased protein binding in the epileptic patients, who were on phenobarbital for a longer period of time before being tested. This was an unexpected result and was true even in those epileptic patients who were only on phenobarbital monotherapy to control their seizure episodes.

Discussion

Phenobarbital is a highly protein-bound drug which is used to control seizures in dogs [10-14]. Understanding the relative percentages of protein binding might be important clinically, since only the free drug appears to be able to cross the blood brain barrier in the adult, although the bound drug may also be able to penetrate this barrier in neonates [15-17]. We were able to demonstrate that phenobarbital can achieve therapeutic blood levels rapidly when given at a 4 mg/kg dose in naïve dogs. While there were some minor side effects noted in these dogs, they were minimal and consisted mainly of PD, PU and sedation. All of these were transitory even during the study. During this initial

		Total	Free	Bound	Free/Bound	% Free	% Bound	TP	Age (yr)	PB Rx (mo)
Mean		26.6	18.2	8.4	3.0	68.9	31.1	6.1	6.9	24.6
SD		18.0	12.3	6.8	2.7	11.3	11.3	0.9	3.4	32.4
Median		20.4	13.5	7.8	2.1	67.4	32.6	6.3	7.0	13.0
Range	Minimum	10.8	8.5	1.1	1.2	53.6	7.4	4.5	0.5	0.5
	Maximum	79.4	57.3	32.8	12.5	92.6	46.4	7.7	14.0	120.0

Total, free and bound phenobarbital levels in serum are expressed as µg/ml. Serum protein (TP) is expressed as g/dl. The age of patients is expressed in years and the length of treatment at the time of the study is expressed in months.

Table 3: Phenobarbital levels, serum protein, age of patient and length of treatment of dogs with epilepsy (n=36).

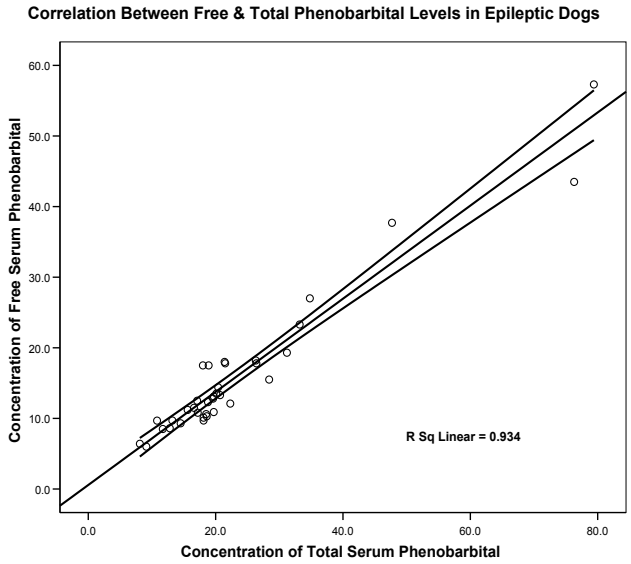

Figure 2: Linear regression correlation between total and free serum Phenobarbital in epileptic dogs (n=36). Outer bars represent the 95% confidence levels around the linear regression line. The value of r was 0.976 indicating a strong correlation between increasing levels of total and free Phenobarbital concentrations.

treatment with phenobarbital, there were consistent free-to-bound parameters and the overall free-to-bound phenobarbital ratio was 0.7. There was more bound drug than free drug. Moreover, a proportion of the bound drug was displaceable. These facts suggest that there is a period of time when the free-to-bound parameters may lead to changes in phenobarbital effective blood levels, since the relative percentage of free phenobarbital may not be reflected by knowing the total level [18]. The free phenobarbital concentration may be altered by as much as 25% based upon alterations in protein binding.

In epileptic patients, we did not examine their phenobarbital levels or free-to-bound parameters until at least 2 weeks from starting phenobarbital. When we examined these samples, we found that the free-to-bound ratio was different than in dogs that were given phenobarbital for only 1 week (8 days). This was not expected. It may be explained by changes in protein binding which phenobarbital is known to induce for other medications and it is speculated that this might even lead to phenobarbital altering its own binding profile [19-22]. Some naturally occurring disease conditions have been shown to alter albumin structure and affect phenobarbital's protein binding [23-25]. Certainly, phenobarbital can lead to changes in certain enzyme systems notably by affecting cytochrome P450 activity [26]. Phenobarbital also alters liver function and bilirubin metabolism [27,28]. These may lead either directly to alter binding or may lead to changes in protein synthesis and composition [29-34].

We did not study the proteins of the epileptic patients to determine if there were qualitative difference, but we did see that there were no quantitative differences between the research dogs and epileptic dogs. This might be necessary to understand the apparent difference between the 2 groups studied in this project. However, this was beyond the scope of the project. Perhaps we could have answered part of the question had we continued treating the research group for a longer period to see if there was a shift from the relatively higher level of phenobarbital binding to the lower state seen in epileptic patients. This should be investigated in the future. Should the difference be shown to continue even after the 2 week interval at which was the first evaluation in epileptic patients, then examination of the serum proteins would be helpful to understand the nature of this difference?

Our epileptic patients did not show an alteration on free-to-bound parameters based upon age which has been shown to influence human free-to-bound phenobarbital levels [35-38]. We also did not see any effect of multiple anticonvulsant drug therapy on free-to-bound phenobarbital [39,40].

When assessing epileptic patients, it is clear that there is a direct linear relationship between total and free phenobarbital serum concentrations [41-43]. As such, there does not appear to be a reason to measure free-to-bound parameters, since the free level can be accurately predicted from the total concentration [44]. On the other hand, since protein binding of phenobarbital in some dogs may not be predictable, it may be worth assessing in refractory patients or during the initial initiation of phenobarbital therapy.

Conclusion

Free-to-bound Phenobarbital concentrations in dogs may vary under certain circumstances; however, the clinical relevance of these changes may be minimal in treating epileptic patients. Even at extreme variations in dosage, free phenobarbital levels were proportional to the total serum concentration.

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Citation: Clemmons RM, Schubert TA, Peters RK, Conrad BL (2013) Free-to-Bound Serum Phenobarbital Levels in Dogs. J Veterinar Sci Technol 4: 143. doi:10.4172/2157-7579.1000143