Serum Protein Binding of Twenty Five Antiepileptic Drugs in a Routine Clinical Setting: A Comparison of Free Non-Protein Bound Concentrations

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Abstract

Objective: As only the free non-protein bound concentration of an antiepileptic drug (AED) crosses the blood brain barrier, entering the brain and producing an antiepileptic effect, knowledge and measurement of the free drug fraction is important. Such data are sparse, particularly for newer AEDs and have arisen from the use of disparate methodologies and setting over the last six decades. We report on the protein binding of 25 AEDs available for clinical use, along with two pharmacologically active metabolites (carbamazepine-epoxide and N-desmethyl clobazam), using standardized methodology and under set conditions.

Methods: The protein binding of the various AEDs was undertaken in sera of 278 patients. Separation of the free non-protein bound component was achieved by using ultracentrifugation (Amicon Centrifree Micropartition System) under set conditions: 500 µL serum volume; centrifugation at 1,000g for 15 minutes and at 25°C). Free and total AED concentrations were measured by use of fully validated liquid chromatography/mass spectroscopy techniques.

Results: Gabapentin and pregabalin are non-protein bound whilst highly bound AEDs (≥ 88%) include clobazam, clonazepam, perampanel, retigabine, stiripentol, tiagabine and valproic acid as well as the N-desmethyl-clobazam (89%) metabolite. The minimally bound drugs (<22%) include ethosuximide (21.8%), lacosamide (14.0%), levetiracetam (3.4%), topiramate (19.5%) and vigabatrin (17.1%). Ten of the 25 AEDs exhibit moderate protein binding (mean range, 27.7-74.8%).

Significance: These data provide a comprehensive comparison of serum protein binding of all available AEDs including the metabolites, carbamazepine-epoxide and N-desmethyl-clobazam. Knowledge of the free fraction of these AEDs can be used to optimize epilepsy treatment.

Key Points

- Epilepsy treatment can be optimized by knowledge of the free fraction of AEDs because only the free non-protein bound component enters the brain so as to exert an antiepileptic effect
- Using a standardised methodology we measured the free fraction of 25 licensed AEDs, including the metabolites, carbamazepine-epoxide and N-desmethyl-clobazam
- The highly bound AEDs (≥ 88%) include clobazam, clonazepam, perampanel, retigabine, stiripentol, tiagabine and valproic acid as well as the N-desmethyl-clobazam (89%) metabolite
- The minimally bound drugs (<22%) include ethosuximide, lacosamide, levetiracetam, topiramate and vigabatrin
- Ten of the 25 AEDs, including the carbamazepine-epoxide metabolite (49.8%) exhibit moderate protein binding (mean range, 27.7-74.8%)
- Gabapentin and pregabalin are not protein bound (0%)

Introduction

The serum protein binding of antiepileptic drugs (AEDs) has a significant impact on their effect as only the free unbound drug is available to pass the blood brain barrier, entering the brain and therefore producing an antiepileptic effect. When binding occurs, typically, AEDs bind reversibly to albumin, the most abundant blood protein, but some binding can occur on α1-acid glycoprotein. AEDs that are considered to be highly bound (i.e. >90%) include phenytoin, valproic acid, tiagabine, perampanel and stiripentol whilst AEDs considered not to be bound include ethosuximide, gabapentin, levetiracetam, pregabalin and vigabatrin (Tables 1-3). The other AEDs are reported to be bound to various degrees between these values.

Routine AED therapeutic drug monitoring (TDM) entails the measurement of serum (or plasma) total concentrations. In most clinical settings, where the relationship between the bound and free unbound components is constant, this approach will suffice.[1] There are, however, clinical settings in which free drug concentrations are significantly higher than expected from total drug concentration measurements; this is particularly the case for those highly bound AEDs.[2] Settings in which this can occur include: uremia, hepatic diseases hypoalbuminemia (e.g. children, the elderly, during pregnancy, post surgery, burns patients, undernourished and HIV infection); and suspected plasma protein binding interactions whereby strongly bound drugs compete.[3-6]

Examples of highly protein bound AED which are susceptible to protein binding impairment are phenytoin and valproic acid. In the case of phenytoin, the free fraction of phenytoin increases, which is followed by increased clearance compensation. The net result is a decreased total concentration level, but normal or higher free levels. Valproic acid shows saturable binding to serum proteins and it may cause binding displacement of other highly protein bound drugs, which does occur when co-administered with phenytoin and also tiagabine.[5,6] TDM of total concentration in this setting would be misleading and only the measurement of free drug serum concentration would relate to the drug's toxicity and efficacy.[5]

Measurement of free drug concentrations can only be undertaken after separation from that that is protein bound and various methodologies have been described including microdialysis, equilibrium dialysis, ultrafiltration, ultracentrifugation and gel filtration and each have their advantages and disadvantages.[4] The most frequently used methods are equilibrium dialysis, used in basic/experimental research and considered to be the gold standard method; microdialysis, used for in vivo studies in humans and animals; and ultrafiltration, the method of choice in a clinical laboratory which provides a routine TDM service because it is convenient to use.[4] Ultrafiltration requires care as ultrafiltrate volume, temperature and ultrafiltration device, centrifugation time and membrane used can impact on the concentration measurement.[7,8]

Monitoring of free levels is cumbersome and technically challenging, as it requires separation of the free fraction and measurement capabilities may not be sensitive enough to measure very low concentrations typically encountered. Ultrafiltration is the preferred method to isolate the free drug, as it is relatively simple, adsorption problems have been overcome with technological improvement[9,10] and precautions can be taken to prevent temperature dependent effects.[18,11] Immunoassays and chromatographic methods are used for AED measurements in TDM units. High performance liquid chromatography (HPLC) and tandem mass spectrometer (MS/MS) coupled together (LC-MS/MS) is the most popular method as it can reliably detect and quantify many different drugs in a single assay.[9,10]

Currently there are at least 25 AEDs licensed for clinical use and these are categorized into first generation, second generation and third generation AEDs (Tables 1-3). Overall, there have been numerous reports describing the serum protein binding of the first generation AEDs and in particular carbamazepine, phenytoin and valproic acid. There is sparse data for the binding of the pharmacologically active metabolite of carbamazepine (carbamazepine-epoxide), clobazam, clonazepam, phenobarbital and primidone (Table 1). There are no reports of the binding characteristics of the active metabolite of clobazam (N-desmethyl-clobazam); indeed there is not even a mention of its binding in the clobazam summary of product characteristics (SPCs). Carbamazepine, phenytoin and valproic acid have more available data as they were the most substantially protein bound AEDs and therefore susceptible to protein binding displacement interactions. Valproic acid also exhibited non-linear pharmacokinetic characteristics consequent to saturable protein binding and this phenomenon attracted many investigations.[10,12] Regarding the second and third generation AEDs, there have been few protein binding studies reported (Tables 2 and 3) and, for some (e.g. brivaracetam, felbamate, perampanel, pregabalin, retigabine, rufinamide stiripentol, tiagabine and vigabatrin) the only data available are those reported in SPCs which are universally vague and lack details as to methodology or methodology conditions. Perhaps another reason for the lack of binding data for the newer AEDs is the fact that most are considered not to be bound to serum proteins or indeed are minimally bound and therefore not associated with pharmacokinetic variability and consequently lack of clinical significance.

Because the available protein binding data have been collected during a 62 year period (1954-2016) using different methodologies and different methodological conditions, a direct comparison between drugs is not possible. We report on the serum protein binding of 25 AEDs, and including the pharmacologically active metabolites, carbamazepine-epoxide and N-desmethyl-clobazam, using a standard methodology (ultracentrifugation; Amicon Cetrifree Micropartition System), under set conditions (i.e. 500 µL serum volume; centrifugation at 1,000g for 15 minutes

and at 25°C). This will allow, for the first time, a comprehensive comparison of AED serum protein binding.

Materials and Methods

Study Population

Data were retrospectively collected from adults attending the specialist epilepsy clinics of the National Hospital for Neurology and Neurosurgery (Queen's Square and Chalfont sites), between November 2009 and August 2016 who had serum samples collected for AED concentration determination, as part of their routine clinical management. Informed consent was not necessary as it only involved data generated as part of routine clinical management.

The samples selected for inclusion were those that had their total AED concentration spanning the quoted reference range for the particular drug. Monotherapy AED samples were preferred but most samples contained polytherapy AEDs. To avoid potential protein binding displacement interactions that could have skewed the data, sera containing concomitant known highly protein bound AEDs (e.g. valproic acid, phenytoin, perampanel and stiripentol) were excluded when the drug of interest was itself highly protein bound. In total, the protein binding of the various AEDs was undertaken in the sera of 278 subjects.

Sample collection

Blood samples (6 mL) were withdrawn at times comprising of pre-dose (i.e. time zero representing a trough sample) and up to 5 hours post AED ingestion. Sampling occurred during the period 09.00-13.00h. After clot retraction blood samples were centrifuged and sera aliquoted into plastic 2 mL microcentrifuge tubes and stored at -80°C until analysis for AED content.

Determination of free concentration

We used an Amicon Cetrifree Micropartition System (Amicon, Stonehouse, UK) to separate in serum the non-protein bound fraction of the various AEDs. Serum (500 µL) was placed on the filter membrane mounted on the sample reservoir and the tube system was centrifuged for 15 minutes using a Sigma 2K15 centrifuge (1,000g) with a temperature setting of 25°C. The AED concentrations in whole serum and in the filtrate were determined and the free fraction of the drug calculated from these values. Free fraction (%) = free concentration/total concentration x 100.

Chemicals

Pure AED compounds were purchased from Sigma-Aldrich (Poole, Dorset, UK) but some were obtained free-of-charge: brivaracetam, lacosamide and levetiracetam (UCB, Chemin du Foriest, Belgium); zonisamide (Elan Pharmaceuticals, UK); N-desmethyl-clobazam (Roche, Welwyn Garden City, UK); felbamate (Schering-Plough, France); tiagabine (Norvo-Nordisk, Sweden); pregabalin (Pfizer Ltd, Sandwich, UK); vigabatrin (Merrell Dow Research, Winnersh, UK); gabapentin (Parke Davis, Cambridge, UK); 10-hydroxycarbazepine (Novartis, Basel, Switzerland); lamotrigine and retigabine (GlaxoSmithKline, Welwyn Garden City, UK); perampanel (Eisai, Japan); rufinamide (Eisai, UK).

Formic acid (analytical grade), and LC/MS grade methanol and acetonitrile were purchased from Sigma-Aldrich (Poole, Dorset, UK). Ultra high quality water was obtained from a Direct-Q system (Millipore, Watford, UK).

AED Analysis

AED free and total concentration analysis was undertaken by LC/MS using fully validated methodologies in routine use within our Therapeutic Drug Monitoring Unit. Validation was based on the most recent versions of the guidelines on bionalytical method validation of the European Medicines Agency and the US Food and Drug Administration. Briefly, an Agilent 1200 series automated LC with an Agilent 6400 series triple quad MS (Agilent Technologies, Stockport, Cheshire, UK) and a HiQ sil C18 column were used. Sera (24 μL) were extracted with 500 μL acetonitrile and prepared for LC/MS analysis by use of a Gilson Quad-Z215 liquid handler (Gilson Instrumentation Services, Luton, Bedfordshire, UK). Calibration curve linearity was observed over the concentration ranges typically seen in routine clinical practice and which spanned the quoted reference range for each AED (Table 4).

Results

Table 4 provides a summary of the characteristics of the various AEDs in alphabetical order. In total, the protein binding of the various AEDs was undertaken in the sera of 278 subjects. For each drug, the mean and range of the total and free concentration and the mean percentage free and total concentration values are shown. The laboratory reference range for each drug is also shown; for most AEDs the serum total concentration measurements were within their depicted laboratory reference ranges. The mean protein binding of N-desmethyl clobazam is 89.2%; almost identical to that of clobazam (90%). The highly protein bound AEDs (≥ 88%) include clobazam, clonazepam, perampanel, retigabine, stiripentol, tiagabine and valproic acid. Only two AEDs appear to be truly non-protein bound, gabapentin and pregabalin whilst the minimally bound AEDs (<22%) include

ethosuximide (21.8%), lacosamide (14.0%), levetiracetam (3.4%), topiramate (19.5%) and vigabatrin (17.1%). Ten of the 26 AEDs can be classified as exhibiting moderate protein binding (mean range, 27.7-74.8%).

The protein binding of valproic acid was determined in the serum of 20 subjects treated with sodium valproate in combination with other AEDs. As shown in Figure 1, the valproic acid free fraction was higher with high serum total concentrations indicating saturable protein binding of valproic acid. However, inter-patient variability in the degree of binding, at any given total concentration value, was considerable.

Discussion

We present the serum protein binding of 25 AEDs that are licensed for clinical use, along with two pharmacologically active metabolites, using a standard methodology under set conditions. Consequently, these data allow, for the first time, a comprehensive comparison of AED serum protein binding. Our data provide interesting findings on the protein binding of AEDs. With regards to the first generation AEDs (Table 1), our data overall concur with previous reports; these include carbamazepine, carbamazepine-epoxide, clobazam, phenobarbital, phenytoin and valproic acid. The 89% protein binding of the pharmacologically active metabolite of clobazam, N-desmethyl clobazam (Table 4), is the first report of its binding and is almost identical to that of the 90% binding of the parent drug, clobazam. For clonazepam, its binding is 90% and similar to clobazam. These data are in contrast to the 47% binding of clonazepam previously reported[18] using spiked human serum albumin and gel filtration and is likely to be due to methodological differences. This may also account for the reported binding of primidone. We found that primidone is 33% protein bound. A previous study using equilibrium dialysis reported a binding of 22%[22] Another study using ultrafiltration reported that primidone is 13.7% bound.[15]

There has been only one previous report of the protein binding of ethosuximide. In a study using a Sephadex column to separate the free from the bound ethosuximide concentration, no significant degree of protein binding, was identified although no actual data were provided.[19] This has lead to the belief that ethosuximide binding is negligible.[39] The SPC for ethosuximide states that ethosuximide is “not significantly bound”. Using the saliva/serum ratio, an indirect method of determining free serum concentration as it is assumed that serum free levels are in equilibrium with saliva values, ethosuximide percentage binding has varied between 0 and 5%.[17] The present study reports a mean percentage bound ethosuximide of 21.8 (Table 4).

We observed that valproic acid binding is saturable within the clinically occurring concentration range so that its pharmacokinetics is non-linear (Figure 1). Additionally, considerable

inter- and intra-patient variability in free valproic acid fraction was observed and all these data are in line with previous reports.[10,12] Due to the changing and unpredictable relationship between total and free concentrations, it would be more rational if valproic acid was monitored by measurement of the free drug.

The protein binding of valproic acid appears to be dependent on sample storage and incubation temperature. Free fatty acid concentrations progressively increase with time and this results in a parallel increase in the valproic acid free fraction. In a study whereby samples were incubated at temperatures varying 4-37°C, at 4°C and room temperature, the increase in free drug fraction was relatively small (18 and 25% at 24h) whereas at 37° it was 22, 34 and 86% at 4, 8 and 24 hours.[40] This time-dependent change in binding capacity may lead to an overestimation of the actual free concentration in vivo. Samples stored at -20°C are stable with no change in either free fraction or free fatty acid concentrations for up to 6 weeks.[11] These considerations did nor pertain in the present study as samples were stored at -70°C and analyzed within 6 weeks of collection. From a practical point of view, if samples are kept refrigerated and the determination of the free fraction is performed within 8 hours of blood withdrawal, changes in valproic acid binding capacity are relatively small. Methodology and sample handling appears to be critical in measurement of valproic acid free fraction, it is therefore not surprising that there is wide variability in reported values where disparate methodologies were used (Table 1).

With regards to the second generation AEDs (Table 2), data on the binding of felbamate, gabapentin, pregabalin, tiagabine and vigabatrin are sparse and the only data available are those reported in SPCs, which lack details as to methodology or methodology conditions, and their binding is stated to be 22-25%, not bound, not protein bound, ~96% and negligible binding, respectively. We found that felbamate is 47.8% bound, tiagabine 97.8% and vigabatrin 17.1%; pregabalin and gabapentin were not bound. These values are broadly comparable with those quoted in the SPCs.

Previous reports of lamotrigine serum protein binding in vivo, as per saliva/serum distribution ratio, was 46-56%.[17] We found a mean percentage bound lamotrigine of 65.7 (Table 4).

A healthy volunteer study of four adults, using ultrafiltration (Amicon Micropartition system; temperature not stated), reported that levetiracetam did not bind to serum proteins to any significant extent. It was stated that binding was <1% in all subjects at 1 hour after drug ingestion and <1% (subject 1), 3% (subject 4) and 6% (subjects 2 and 3) at 12 hours after administration.[28] These data are in line with what we found whereby levetiracetam mean percentage bound concentration was 3.4 (Table 4).

A study of 17 subjects with epilepsy used three different methods to investigate the protein binding of 10-hydroxycarbazepine (the pharmacologically active metabolite of oxcarbazepine): equilibrium dialysis (37°C), ultrafiltration (EMIT free level system-SYVA; ambient temperature) and saliva/serum ratios.[29] Despite the disparate methodologies and conditions, 10-hydroxycarbazepine binding by the three methods was almost identical and was respectively (mean ± sd) 40 ± 4%, 45 ± 8% and 41 ± 12%. Furthermore, these data concurred with that of a study of six people with trigeminal neuralgia whose 10-hydroxycarbazepine binding was 39.0±6.0.[30] The SPC quotes a value of ~40%.

In a review,[31] it was noted that topiramate was 9-17% protein bound and the SPC states a binding of 13-17% whilst saliva/serum ratio data report topiramate binding to range 0-37%.[17] Noteworthy is that there is a low capacity binding site for topiramate in/on erythrocytes that is saturable above serum concentrations of 4 mg/L (Topamax – SPC). We report a mean percentage bound topiramate of 19.5 (Table 4).

Zonisamide protein binding was investigated using ultrafiltration (Amicon microparitition system; temperature not stated) in six health volunteers and a mean ± sd binding of 39.4 ± 1.2 was observed[32] and this concurred with a value of 40-50% quoted in the SPC. Using the same methodology as in this previous study and at a temperature of 25°C, we found a mean percentage binding of 43.7 (Table 4).

There is almost no binding data for the third generation AEDs (Table 3). Indeed for brivaracetam (≤ 20%), retigabine (~80%) and stiripentol (99%) the only data available are those reported in SPCs. We found binding of 35% for brivaracetam, 88% for retigabine and 96% for stiripentol and these data are overall comparable with those quoted in the SPCs.

The protein binding of eslicarbazepine is reported in the SPC to be <40% whilst eslicarbazepine binding is stated in a review to be 30%.[33] Neither publication states the methodology used. We found a mean percentage bound eslicarbazepine of 43.8 (Table 4).

The SPC for lacosamide, which does not stipulate methodological details, reports that lacosamide binding to serum proteins is <15%. In contrast, a study using microfiltration (Amicon Centrifree Micropartition system) with centrifugation at 25°C, observed that protein binding in serum collected from 48 subjects with epilepsy was 91±4% whilst that calculated from saliva measurement was 87 ± 4%.[34] Using saliva/serum concentration ratio data, a study reported that lacosamide was 2% protein bound after tablet ingestion whilst binding was 9% after syrup administration; overall, therefore the value was considered to be <10%.[36] We observed a mean percentage bound lacosamide of 14.0 (Table 4) and is in accord with that of the SPC and the data

from other studies[35,36] but in contrast to another.[34] The disparate results of this study[34] are perplexing as the same methodology used in the present study was used.

In a review[37], it was reported that perampanel in vitro plasma protein binding ranged 95-96% (data provided by Eisai). These data are in line with that of ~95% binding quoted in the SPC. The present study reports a mean percentage bound perampanel of 97.6 (Table 4).

Lastly, in an extensive review[38], it was reported that rufinamide "in vitro protein binding to albumin ranges 26.2- 34.8%" – these data are referenced as "Eisai data on file" and are in line with that of 34% binding quoted in the SPC. The present study reports a mean percentage bound rufinamide of 27.7 (Table 4).

The ultrafiltration methodology used to prepare the free fraction of the different AEDs in the present study needs mention. Ultrafiltration is considered to be the method of choice in clinical laboratories which provides a routine TDM service and this is indeed the practice choice within our TDM Unit; its key feature is convenience of use.[4] Ultrafiltration requires care as a number of factors can impact on the concentration measurement.[7,8] In an extensive study, the effect of different temperatures on the protein binding of phenytoin (n=131), carbamazepine (n=121) and phenobarbital (n=36) taken as monotherapy was assessed.[8] The Amicon Centrifree Micropartition system was used and centrifugation was for 20 minutes at 1,000 g. The free fraction of all three AEDs was observed to be temperature dependent: for phenytoin the mean value was 0.1 (25°C) and 0.15 (37°C); for carbamazepine the mean value was 0.26 (25°C) and 0.30 (37°C); and for phenobarbital the mean value was 0.60 (25°C) and 0.63 (37°C). Interestingly, a previous study using the technique of ultrafiltration investigated the effect of temperature (4°C, 26°C and 40°C) on the protein binding of phenobarbital observed no significant effect and subsequently undertook all further experiments at room temperature and reported that phenobarbital is 40% bound.[20] We report a mean percentage bound phenobarbital of 47.8 and is in line with essentially all previous reports (Table 4). It was reported that the unbound fraction of phenytoin was 0.073 ± 0.007 at 25°C and 0.117 ±0.008 at 37°C when studied by equilibrium dialysis and this concurs with subsequent findings.[8]

Other variables that can impact on free fraction measurements is that of diurnal rhythms of drug distribution. For example, a study of 12 subjects with epilepsy using ultrafiltration (Millipore Corp, Freehold, NJ; temperature not stated) in which samples from different times of the day were used found that valproic acid free drug (%) was 10.6 ±1.4 in the morning; 12.6 ±2.2 in the afternoon and 12.0 ±2.3 in the evening. Similar diurnal oscillations were observed for phenytoin, primidone, phenobarbital and carbamazepine.[15]

Conclusion

We report on the serum protein binding of 25 currently available AEDs using a standard methodology under set conditions. The pharmacologically active metabolites, carbamazepine-epoxide and N-desmethyl-clobazam were also included and these data allow, for the first time, a comprehensive comparison of AED protein binding. The AEDs can be divided into those that are highly protein bound (≥ 88%) and include clobazam, clonazepam, perampanel, retigabine, stiripentol, tiagabine and valproic acid. Gabapentin and pregabalin are non-protein bound whilst the minimally bound AEDs (<22%) include ethosuximide (21.8%), lacosamide (14.0%), levetiracetam (3.4%), topiramate (19.5%) and vigabatrin (17.1%). Ten of the 25 AEDs can be classified as exhibiting moderate protein binding (mean range, 27.7-74.8%).

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Disclosures:

We confirm that we have read the Journal's position on issues involved in ethical publications and affirm that this report is consistent with those guidelines. JWS has received research support from Eisai, UCB and GSK and personal fees from Eisai, UCB, Lundbeck and Teva, outside this work. For the remaining authors none were declared.

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Figure 1 legend:

Unbound valproic acid fraction (%) as a function of the total serum concentration in 20 patients with epilepsy receiving multiple antiepileptic drug (AED) therapy with sodium valproate. Patients co-prescribed highly protein bound AEDs were excluded from this comparison and thus the potential for protein binding displacement interactions was not a consideration.