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BINDING OF l-α-ACETYLMETHADOL AND ITS METABOLITES TO BLOOD CONSTITUENTS

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Abstract—The distribution *in vitro* of (-)l-α-acetylmethadol (LAAM) in human blood constituents was studied. In concentrations close to those found in humans who are maintained on LAAM (0.35 nmole/ml serum), the drug was distributed almost evenly between plasma proteins and red blood cells. At similar concentrations of the drug in plasma alone, over 80 per cent was bound to protein. The strength of binding to proteins was very weak, as demonstrated by our inability to obtain a protein–LAAM complex by conventional Sephadex G-200 column chromatography. However, equilibration and elution of the column with buffer containing 0.35 nM drug, using [^{3}H]LAAM as tracer, allowed the identification of a LAAM–protein complex. The fraction responsible for the bulk of LAAM binding in serum was identified as an α-globulin with a molecular weight of about 400,000. Equilibrium dialysis experiments showed that the role played by albumin in the binding of LAAM was insignificant. The binding of LAAM to serum proteins was highly reversible, as attested by the displacement of [^{3}H]LAAM from its binding sites by unlabeled drug. The major metabolites of LAAM, noracetylmethadol and dinoracetylmethadol, were also weakly and reversibly bound by serum proteins and competed with LAAM for protein binding sites. A Scatchard plot, after equilibrium dialysis of various concentrations of LAAM against human serum, indicates that the maximum specific binding of drug was 8.2 nmoles/ml serum. These data suggest that, assuming at least one binding site per protein molecule, the binding occurs to a protein of very low concentration (about 1 mg/ml) in plasma. This is consistent with the data that suggest an insignificant role of human serum albumin in the binding of LAAM and the identification of a very high molecular weight protein as the possible binding entity. The data suggest that LAAM, its metabolites, and methadone compete for the same protein binding sites and that the binding capacities of plasma for both LAAM and methadone are of the same order of magnitude. The results failed to show any cooperativity on the plasma protein binding of LAAM, its metabolites or methadone.

A complete study of the physiological, pharmacological, biochemical and physico-chemical properties of the narcotic analgesic l-α-acetylmethadol (LAAM), a long-acting derivative of methadone developed originally as an analgesic and having a opiate-like profile [1], would be useful prior to its large-scale introduction in the treatment of heroin addiction in humans.

A diversity of reports indicates that *in vivo* the narcotic analgesic LAAM is converted to two major metabolites, namely α-acetylmethadol (nor-LAAM) [2] and α-acetyldinormethadol (dinor-LAAM) [3]. Both metabolites have been found to remain in the circulation for prolonged periods [4], and there is existing evidence that a significant portion of the activity of LAAM is due to its metabolites [3, 5]. LAAM and its metabolites have been found to bind to the opiate receptors of rat brain [6].

Despite the fact that the binding of drugs to plasma proteins is well established as an important variable in the pharmacological and therapeutic activities of medicinal agents, to our knowledge no studies have been reported on the binding and distribution of LAAM and its major metabolites to blood constituents.

In this work we report the distribution of LAAM between red blood cells and plasma proteins. By using the techniques of gel filtration and equilibrium dialysis, we studied *in vitro* the nature, extent and reversibility of the binding of LAAM and its metabolites to plasma proteins. We also identified a LAAM binding fraction present in low (1–2 mg/ml) concentration in plasma and characterized it as a high molecular weight α-globulin.

MATERIALS AND METHODS

Drugs. [^{3}H]LAAM, tritiated in carbon 2 of the heptyl chain sp. act. 40.3 mCi/mmole; [^{3}H]nor-LAAM, tritiated in the *o,o'* carbon atoms of the phenyl rings, sp. act. 1.40 Ci/mmole; [^{3}H]dinor-LAAM, tritiated in the *o,o'* carbon atoms of the phenyl rings, sp. act. 94 mCi/mmole; and [^{3}H]methadone, tritiated in the *o,o'* carbon atoms of the phenyl ring, sp. act. 16.7 Ci/mmole, were obtained as their hydrochloride salts from the Research Triangle Institute (Research Triangle Park, NC, U.S.A.) as authorized by the National Institute on Drug Abuse. All the compounds were over 95 per cent pure as determined by radioscan after thin-layer chromatography. The above named unlabeled compounds were also supplied in crystalline form by the same organization.

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Materials. All chemicals were reagent grade except cesium chloride (CsCl), which was optical grade ultrapure. Sephadex G-200 (40–120 μm, 30–40 ml bed vol/g dry gel) was obtained from Pharmacia (Uppsala, Sweden). Gravity flow columns (1.5 × 100 cm, 1.5 × 60 cm, and 5.0 × 100 cm) were used for gel filtration chromatography (Glenco Scientific, Houston, TX, U.S.A.)

Methods. The gel filtration chromatography was carried out at a room temperature of 20 ± 3°. Absorbancies were detected with an ISCO UA-5 absorbance monitor and recorder (Instrumentation Specialties Co., Lincoln, NE, U.S.A). The eluant was collected in tubes in an automatic fraction collector. Radioactivity was counted in a Beckman liquid scintillation counter (Beckman Instruments, Palo Alto, CA, U.S.A.), and quench was corrected by external standardization.

Blood was collected in disodium edetate vacutainers (Becton–Dickinson, Rutherford, NJ, U.S.A.), from non-fasting, apparently healthy individuals with no current or past history of narcotic abuse. When serum was desired, blood samples were collected in the absence of anticoagulants, allowed to clot at room temperature and centrifuged immediately thereafter. The serum was removed and stored at 4°. For larger amounts of plasma, outdated blood from the institution's blood bank was used.

The time course of binding of the different drugs to serum proteins was determined by dialysis. Equilibrium dialysis was used to determine per cent binding of drugs to protein. Bound/free ratios were calculated at various concentrations of drug for Scatchard plots [7].

The basic apparatus for the dialysis has been described before [8] and consisted of a magnetic stirrer, a stirring bar, a dialysis bag, a short glass tube (inserted in a rubber stopper) to which the dialysis bag was fastened with surgical silk, and a 250-ml graduated cylinder to hold the dialyzing solution. The opening made by the glass tube allowed the serial removal of serum aliquots at various time intervals. The dialysis tubing was prepared by heating for 1 hr at 80° in the presence of 1% EDTA and soaking in water with 0.1% EDTA until used. Dialysis was carried out at 20 ± 3°. At the start of the run, 200 ml of the buffer (150 mM NaCl, 1 mM KH_2PO_4, pH 7.35) were added to the graduated cyclinder, and the desired amount of drug was added from stock 1 mM solutions. Two milliliters of serum were pipetted inside the bag and equilibration was allowed to proceed.

For partial characterization of the LAAM-binding fraction of plasma, chromatographic columns (5 × 100 cm) were used to fractionate 15 ml of plasma or serum by gel filtration. The desired fractions were collected, enough CsCl was added to bring their densities to 1.21 g/ml, and they were centrifuged for 30 hr at 286,000 *g* in a Beckman model L3-40 ultracentrifuge using a model SW41T1 swinging bucket rotor. The supernatant fluid from the tubes was removed and discarded. The lowest 1-ml fraction in the bottom of the tubes was collected, dialyzed exhaustively against 0.1 M NH_4HCO_3, and lyophilized. The electrophoretic mobility of this material was characterized by Sepraphore polyacetate electrophoresis and its molecular weight estimated by sodium dodecylsulfate (SDS) gel electrophoresis. Gels were prepared as described by Maizel [9] and molecular weights were estimated according to Weber and Osborn [10]. Proteins of known molecular weights (Sigma Chemical Co., St. Louis, MO, U.S.A.), were used as standards in the calibration curve for molecular weight determination.

Quantitative protein determinations were made by Sutherland's [11] modification of the Folin reaction. Spectrophotometric measurements were done in a model 635 Varian UV-Vis spectrophotometer (Varian Associates, Palo Alto, CA, U.S.A.).

RESULTS

Distribution of LAAM in blood constituents. The results presented in Table 1 indicate that in whole blood, LAAM is distributed nearly evenly between red blood cells and plasma: the results are consistent for the six subjects studied. The subjects used for this study had not been recently exposed, to our knowledge, to LAAM or any other opiate-like drugs, and significant differences could occur in individuals exposed to LAAM or analogous drugs. Evidence of the weak nature of the binding of LAAM to plasma protein was obtained by Sephadex G-200 gel filtration, as illustrated in Fig. 1. The elution pattern indicated that, even when the serum proteins were allowed to equilibrate for 18–24 hr with [^{3}H]LAAM, the binding was easily reversible since almost all the drug eluted after the proteins. The radioactivity coincided with the elution of other small molecules present in plasma which absorb at 280 nm and that elute with the total volume (or volume accessible for diffusion) of the chromatographic columns. Similar results were obtained with other plasma samples (N = 4). This chromatographic behavior is in marked contrast to highly lipophilic drugs, such as the cannabinoids, that are strongly bound to plasma proteins and elute from the column together with the proteins*. Since the metabolites of LAAM (nor-LAAM and dinor-LAAM) have been found to last for periods of over 48 hr in plasma, we also performed a gel chromatographic study of these drugs after equilibration with plasma to ascertain whether their chromatographic behavior was different from that of LAAM. The elution behavior of nor-LAAM and dinor-LAAM did not differ from that of LAAM.

Equilibrium dialysis. Our experimental approach to equilibrium dialysis was to allow the plasma proteins to accumulate the drug until saturation. The time taken to attain saturation was determined by removal of aliquots from the dialysis bag and dialyzing buffer at various time intervals until the ratio of bound/free drug reached a plateau. The plateau for LAAM was attained 24 hr after start of the dialysis, as can be seen in Fig. 2. Bound/free ratios were also calculated from samples removed at 36 hr or longer. A 5-fold dilution of the serum reduced the maximum bound/free ratios from 4.0 to approximately 2.5.

Reversibility of the binding of LAAM. Figure 3 shows the reversibility of the binding of LAAM.

* B. R. Martin and E- Toro-Goyco, unpublished data.

Table 1. Distribution of [^{3}H]LAAM between red blood cells and plasma in human subjects*.

Subject	Hematocrit	Blood†	Plasma†	RBC†	RBC/plasma
1	47	5.86	6.44	5.22	0.81
2	44	5.85	5.65	6.10	1.08
3	43	5.16	5.31	4.97	0.94
4	43	5.66	5.70	5.61	0.98
5	43	5.28	5.76	4.64	0.81
6	43	4.99	5.47	4.41	0.81

* Two milliliters of heparinized blood were incubated in duplicate with [^{3}H]LAAM (7 μM) for 1 hr at 37°. Blood (0.5 ml) was removed from each tube and counted for radioactivity. The remaining blood was centrifuged, and plasma (0.5 ml) was removed for counting. Binding to RBC was calculated from the following relationship: d.p.m./ml RBC = [d.p.m./ml blood-d.p.m./ml plasma × (1-hematocrit)] ÷ hematocrit.

† Concentration of [^{3}H]LAAM expressed as nmoles/ml.

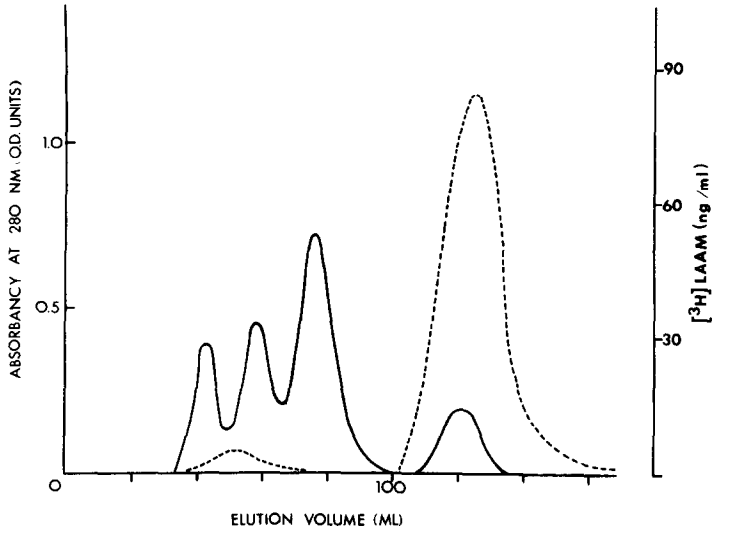

Fig. 1. Sephadex gel filtration chromatography of human serum (2 ml) incubated with [^{3}H]LAAM (2 μg) overnight prior to chromatography. Dimensions of the column were 90 × 1.5 cm; Key: absorbancy at 280 nm (———); and [^{3}H]LAAM (– – – –).

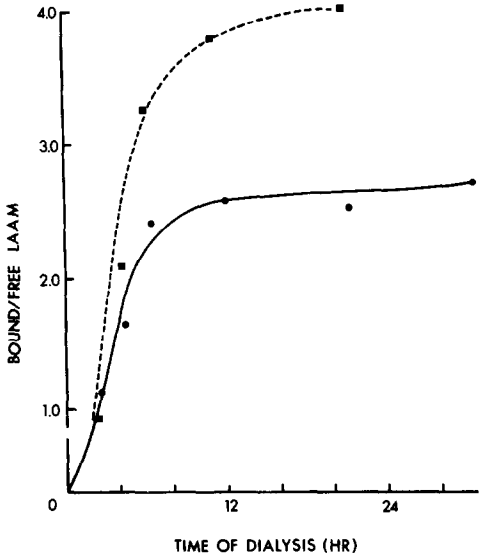

Fig. 2. Time course for the saturation of [^{3}H]LAAM (0.35 nmole/ml buffer) with serum proteins. Equilibration was allowed to proceed as described in the text. Key: 2 ml of undiluted serum (– – – –); and 0.4 ml of serum diluted to 2.0 ml (———).

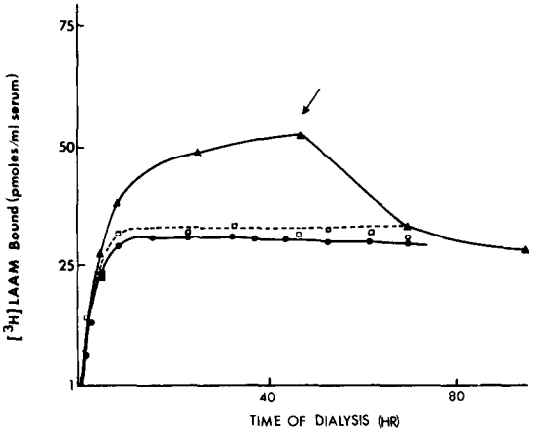

Fig. 3. Time course for the equilibration of [^{3}H]LAAM with serum (2 ml) at various ratios of labeled to unlabeled drug. A ratio of 1:5 is indicated by closed triangles (▲——▲), with a starting concentration of [^{3}H]LAAM of 0.07 nmole/ml buffer; a ratio of 1:25 is indicated by open squares (□–□–□); a ratio of 1:50 is indicated by closed circles (●—●). An arrow (↓) indicates a shift to 1:50 in the labeled to unlabeled ratio after equilibrium had been attained.

Table 2. Comparison of the binding by human serum of various opiate-like analgesics*

Drug	Bound/Free (24 hr)	Bound/Free (48 hr)
[^{3}H]Methadone	3.66	2.48
[^{3}H]LAAM	4.06	3.14
[^{3}H]Nor-LAAM	3.96	4.22
[^{3}H]Dinor-LAAM	3.19	3.83

* Binding was determined by equilibrium dialysis. Each drug was added to the buffer (1 nmole/ml) and the amount bound by 2 ml of serum measured at 24 hr and 48 hr.

When the drug was incubated with plasma, and the ratio of labeled:unlabeled drug was 1:5, serum proteins bound a significantly higher amount of radioactivity than when lower ratios (1:25 and 1:50) were used. After the addition of a 45-fold excess of unlabeled drug, thus decreasing the labeled to unlabeled ratio from 1:5 to 1:50 (as indicated by the arrow in Fig. 3), the amount of radioactivity bound diminished, reaching the values of radioactivity bound when the dialysis was started with a labeled to unlabeled ratio of 1:50. An identical effect was observed when LAAM (labeled to unlabeled ratio of 1:5) was incubated with serum in the presence of an excess of either unlabeled nor-LAAM or dinor-LAAM. The addition of a 20- and 45-fold excess of either unlabeled metabolite at the beginning of the dialysis decreased [^{3}H]LAAM binding by almost 50 per cent. When the 1:5 ratio of [^{3}H]LAAM/LAAM was shifted to 1:50 with nor-LAAM and dinor-LAAM (after 48 hr), a decrease in binding of [^{3}H]LAAM similar to that in Fig. 3 was observed. It can be inferred from these results that the binding of LAAM is highly reversible and that the metabolites compete for the same binding sites.

The reversal of the situation, the displacement of [^{3}H]dinor-LAAM and [^{3}H]methadone from their binding sites by LAAM, was also studied. After a 24-hr equilibration of serum with the radiolabeled drugs (1 nmole/ml buffer), addition of a 50-fold excess of unlabeled LAAM caused their displacement from the binding sites, as shown by a decrease in the amount of bound radioactivity per ml plasma.

The data presented in Table 2 show that the binding affinities for LAAM, its major metabolites, nor-LAAM and dinor-LAAM, and methadone are of the same order of magnitude when their respective concentrations in plasma are identical (1 nmole/ml). We also found that when [^{3}H]LAAM, [^{3}H]nor-LAAM, [^{3}H]dinor-LAAM and [^{3}H]methadone were added to serum at a concentration of 1 nmole/ml, a 5- to 10-fold excess of all unlabeled drugs studied was equally effective in displacing the radiolabeled ligand. These results are also consistent with the fact that binding is weak and reversible and that the four drugs studied here compete for the same binding sites. The degree of drug–drug competition has not been established at lower ligand concentrations.

Identification of the LAAM-binding fraction. Our failure to isolate and identify a protein–LAAM complex by gel filtration led us to simulate conditions that favor equilibrium between drug and protein. Equilibrium of a chromatographic column with a buffer containing a given concentration of drug (in this case LAAM) favors the possibility of identifying a LAAM–protein complex no matter how weak it may be. This is true for any ligand, provided appropriate concentrations of the ligand are found in the eluting buffer.

A typical elution pattern for such an experiment is illustrated in Fig. 4. We are able to identify a protein fraction that binds LAAM. This fraction eluted between the first and second protein peak of the chromatogram shown in Fig. 1. Having identified the probable elution behavior of the protein fraction binding LAAM, we fractioned serum in the same column and removed three fractions corresponding to three different points in the chromatogram. These three corresponded to the LAAM-binding protein, the immunoglobulins, and albumin. An aliquot of each fraction was analyzed for protein content, and the remainder was used for the determination of LAAM binding by equilibrium dialysis. The results of this experiment are summarized in Table 3. The fraction binding the most radioactivity in the equilibrium experiment in the chromatographic column proved to be the one having the highest affinity for LAAM. The immunoglobulins, as expected, did not bind LAAM. Surprisingly, albumin plays a minor role in the binding of LAAM.

Further characterization of the LAAM-binding fraction. Further characterization of the LAAM-binding protein indicates that it had the electrophoretic mobility of an α-globulin. On SDS gel electrophoresis, because of dissociation of polypeptide chains in the several proteins comprising this fraction, several different bands of varying molecular

Table 3. Binding of LAAM to plasma fractions from a Sephadex column

Fraction No.	Component	Protein (μg/ml)	LAAM bound (ng/ml)	Drug (ng/μg protein)
32	α-Globulin	94	75	0.76
41	γ-Globulin	260	0	0
52	Albumin	274	23	0.08

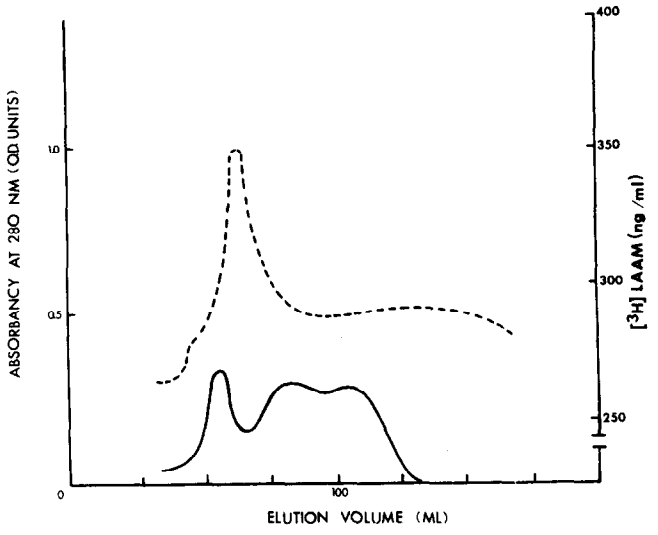

Fig. 4. Elution pattern of human serum equilibrated overnight with [^{3}H]LAAM (0.07 nmole/ml); labeled:unlabeled ratio, 1:5) and chromatographed in Sephadex G-200 with a buffer containing LAAM (0.35 nmole/ml). Dimensions of the column were 60 × 1.5 cm. Key: absorbency at 280 nm (———); and [^{3}H]LAAM (– – –).

weights were observed. Two of the sharpest bands in the gel, however, corresponded to particles of molecular weights close to 400,000.

The Scatchard plot presented in Fig. 5 indicates that 1 ml of plasma binds specifically 8.2 nmoles of LAAM. These results corroborate the previous data which indicate that albumin plays an insignificant role in the binding of LAAM and that the drug must be bound by a protein of low concentration in plasma.

We also performed equilibrium dialysis experiments using methadone as a ligand to determine its binding behavior in plasma. A Scatchard plot (data not included) yielded results very similar to those found for LAAM.

DISCUSSION

Several factors determine the rate at which a drug leaves the circulation. Among these are the drug's partition coefficient (ratio of solubility in lipid to solubility in water), molecular weight, and state of aggregation in plasma. For drugs like LAAM and its metabolites, which are of low molecular weight and water-soluble, these factors alone would favor their rapid disappearance from plasma. The remaining factor, state of aggregation, may be very important in determining their long-lasting presence. Our results show that, at the drug levels found in humans treated with these drugs (100–1000 ng/ml serum) [4, 12], the fractional binding and the partition coefficient could be important parameters in determining their continuous presence in plasma. In the absence of an active renal process for the clearance of the drug from plasma, only glomerular filtration will account for their removal in urine. If the fractional binding is high, as shown by the results presented in Fig. 5 and Table 2, the amount of drug available for glomerular filtration rate is diminished significantly. Despite a high fractional binding, strength of binding is weak. This last factor favors rapid removal from plasma. Unfortunately, there is no information available as to the the fate of LAAM and its metabolites after glomerular filtration. The indirect evidence at hand (long duration in spite of weak plasma binding) suggests that re-entry of the drug into the circulation by reabsorption cannot be discarded as a possibility.

It has been reported [13] that, in subjects receiving LAAM, 24-hr plasma levels show the simultaneous presence of LAAM and its two major metabolites, but the bulk consists of the metabolites. Our results suggest that this can be accounted for by the displacement of LAAM from its binding sites by its metabolites. Probably the higher lipid:water partition ratio of LAAM* contributes to its removal from plasma in preference to its metabolites.

In the evaluation of protein–drug interactions, in addition to considering protein–drug affinities and

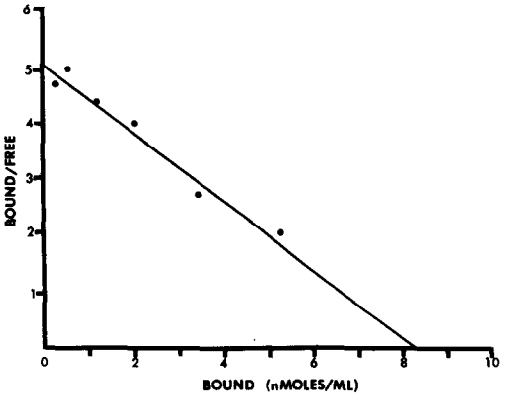

Fig. 5. Scatchard plot for the binding of LAAM by human plasma. The data are an average of four experiments.

* E. Toro-Goyco, unpublished data.

the fractional binding of a drug, a remaining important consideration is the size of the protein to which it is bound. For a bound drug the rate of transcapillary passage will be determined by the rate of transcapillary passage of the protein. Capillaries of various organ systems show a wide variation in their permeability to substances. For example, those of the liver allow macromolecules to permeate easily. We can expect a metabolizable drug bound to albumin to be easily permeable to the hepatic cells. The opposite will be true if the drug is bound to a protein of larger size. The evidence presented here suggests that transcapillary passage of LAAM and its metabolites can be expected to be slower because of the binding to a high molecular weight protein (Fig. 4 and Table 3). However, the almost equal distribution between plasma and red blood cells suggests a very similar pattern of distribution in body tissues.

Taking into consideration all these factors we must conclude that our evidence is not strong enough to justify a statement asserting that the long-lasting presence of LAAM and its metabolites in plasma can be explained on the basis of the strength, nature, and magnitude of their binding to plasma proteins.

An unexpected finding in this work has been the weak role played by albumin in the binding of these drugs. Albumin plays the most important role in drug binding by plasma proteins and is usually taken as a model protein for drug studies. Olsen [14] has shown that methadone binds to albumin, but our data show that this binding is readily reversible. It has been reported previously that methadone binds mostly to a globulin fraction [8]. The same author reports a cooperative phenomenon for the binding of the drug by albumin, that is, a higher binding fraction with increasing concentrations of the drug. We used serum for our experiments (containing about 40 mg/ml albumin) but were unable to detect such cooperativity with any of the drugs.

Being aliphatic amines, LAAM and its metabolites are protonated at physiological pH. It would be logical to expect binding to the albumin molecule because of its large number of carboxyl groups available to form salt-like linkages. This is not the case since binding occurs preferentially to a globulin. It is possible that another negatively charged group (possibly a sulfate) may be forming a salt-like linkage with the amine, although this remains to be documented.

The experimental approach used to identify a protein–drug complex (Fig. 4) is very helpful in cases where protein–drug complexes are difficult to identify because of the weakness of the association. This approach has been used before to identify plasma protein–calcium complexes in human serum [13]. The results shown in Fig. 5 (Scatchard plot) confirm the findings shown in Fig. 4. It shows that 1 ml of serum (or plasma) is capable of specifically binding about 8.2 nmoles of LAAM. If we assume a minimum of one binding site per protein molecule, we conclude that albumin binding is a very likely possibility since 1 ml of plasma contains close to 600 nmoles of albumin. On the other hand, if we suppose the binding globulin to be a very scarce protein in plasma, as suggested by the results in Figs. 1 and 4, and this protein to be a high molecular weight entity of around 400,000 as calculated from SDS gel electrophoresis experiments, we can safely conclude that a protein with a concentration of 1–2 mg/ml plasma could very well be the binding protein. Small changes in the concentration of this binding protein, without a significant change in total plasma proteins, could cause marked changes in the LAAM-binding capacity of the blood.

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