Abstract

KAREN YEOWELL O'CONNELL. Development of a Method for the Isolation and Quantitation of Albumin for Use in the Analysis of Albumin Adducts. (Under the direction of STEPHEN RAPPAPORT)

In order to improve exposure assessment in epidemiological studies, alternatives to traditional methods such as air sampling are under investigation. The ability to detect individual exposures through the use of biomarkers is thought to be a way to improve the accuracy and specificity of exposure assessment. Protein adducts are biomarkers which show potential for giving more specific information about exposure. Since 1985, investigators have been validating methods that measure adducts of potentially genotoxic species with serum albumin. One of the major difficulties encountered by these researchers is the isolation and quantitation of albumin. This research involved testing two procedures for the isolation of albumin from plasma (affinity chromatography and ammonium sulfate precipitation), two methods for desalting the isolated albumin (gel filtration and dialysis), and two methods for albumin quantitation (Bradford assay and absorbance at 280nm). Based on purity of albumin obtained (as judged by SDS-PAGE), comments in the literature, and applicability of the methods to screening large numbers of plasma samples, a rapid method of albumin isolation and quantitation was developed for use in analyzing protein adducts.
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Introduction

Biological markers are becoming increasingly important tools for improving the validity of epidemiologic research. Biomarkers are cellular, biochemical, or molecular alterations that are measurable in biological media such as tissue, urine, or blood (Hulka, 1990). Biomarkers can be used to monitor individual exposure (as internal dose or molecular dose), early biological response, or to give an indication of individual susceptibility. DNA and protein adducts are examples of biomarkers which can be used in lieu of external exposure measurements (such as ambient air concentration). Since adducts can indicate the amount of xenobiotic which has been absorbed and, in many cases, metabolized by an individual, their use can yield great improvements in the sensitivity and specificity of individual assessments of exposure (Hulka, 1990).

The use of protein adducts as a potential surrogate for DNA adducts was first proposed by Ehrenberg et al. in 1974. Although protein adduct formation is not typically associated with the carcinogenicity of genotoxic species, protein binding is thought to be proportional to DNA binding for a number of compounds (Skipper and Tannenbaum, 1990). Blood proteins have a number of advantages in that they are available in large quantities and are relatively easy to obtain. In addition, protein adducts are not repaired (unlike most DNA adducts), so they can be used to estimate long term exposures.

Hemoglobin has been the primary protein used for measurements of
protein adducts due to its abundance (~150 mg Hb/mL blood), ease of isolation, and long residence time in the body (~120 days). However, since 1980, investigators have also been looking at adducts of plasma proteins, and since 1985, albumin (Alb) adducts. The information gained from studying Alb adducts can be used either in addition to or in lieu of that obtained from hemoglobin adducts.

Although Alb is present in blood at a lower concentration than hemoglobin and has a shorter half life than hemoglobin (20-25 days) (Skipper and Tannenbaum, 1990), higher concentrations of Alb adducts are possible for several reasons (Henderson et al., 1989). Reactive species do not have to penetrate the erythrocyte membrane in order to form a covalent adduct, as they do for hemoglobin. In addition, Alb is synthesized in the liver, where much of the metabolic activation for most xenobiotics occurs (Sipes and Gandolfi, 1991). Consequently, binding of the activated metabolites to Alb in the liver is theoretically possible (Skipper and Tannenbaum, 1990). Alb adducts have been successfully detected for a number of xenobiotic compounds, including the reactive metabolites of styrene (Rappaport et al., in press), benzene (Bechtold et al., 1992), 4-aminobiphenyl (Skipper et al., 1985), monochloroacetic acid (Kaphalia et al., 1992), dichloroacetate (DCA), trichloroacetate (TCA), trichloroethylene (TRI) (Stevens et al., 1992), 2-amino-3-methylimidazo[4,5-f]quinoline (IQ) (Turesky et al. 1987), aflatoxin-B$_1$ (Autrup et al., 1991; Gan et al., 1988; Groopman et al., 1992; Sabbioni et al., 1987, 1990a; Wild et al., 1990), aflatoxin-G$_1$ (Sabbioni and Wild,
1991), benzo[a]pyrene (Lee et al., 1988; Sherson et al., 1990), and 4,4'-methylenebis(2-chloroaniline) (MOCA) (Cheever et al., 1991).

Although measurement of Alb adducts can be a useful technique to assess exposure to a variety of different compounds, no standard method exists for isolating and quantifying plasma Alb. All of the techniques currently in use have significant disadvantages associated with them. These include lack of purity of the Alb, potential loss of the adduct during the isolation, excessive time required to perform the procedure, and possible inaccuracies in Alb quantitation. The goal of this research was to develop a simple, rapid method for the isolation and quantitation of Alb from large numbers of blood samples.

This report reviews the structure and function of Alb, the particular ways Alb is being isolated for use in the analysis of protein adducts, and the major procedures historically used to isolate and quantitate Alb. A brief description of styrene and how the isolation and quantitation methods described in this report have been applied to the detection of styrene adducts of Alb is also included. The procedures that were tested to isolate, purify, and quantify Alb are described in detail. This report also explains the rationale behind choosing the final procedure and its advantages and disadvantages in comparison to other available methods.

Abundance, Structure and Function of Albumin

Alb is the most abundant protein in plasma, with a concentration of about 35-45 mg/mL plasma in humans (Birke et al., 1979) and 16 mg/mL
blood in rats (Ringler et al., 1979). Alb represents about 52% of plasma proteins, with the immunoglobulins making up 11% and fibrinogen about 4% (Schultze and Heremans, 1966). When Alb is isolated from plasma the globulins are the primary proteins from which Alb needs to be separated.

Human Alb consists of 584 amino acid residues and has a calculated molecular weight of 66,248. Rat Alb consists of 575 residues and has a molecular weight of 64,616 (Peters, 1975). The amino acid sequence includes 35 cysteine residues, which gives rise to 17 disulfide bridges and a single free sulfhydryl group at position 34. These bridges maintain Alb in 9 loops, which are evenly distributed over 3 domains (Rothschild et al., 1988). There is a lone tryptophan at residue 214 (Andersson, 1979), and the protein is rich in the charged residues, aspartic acid, glutamic acid, lysine, and arginine (Rothschild et al., 1988) which serve to give the protein a net charge of -10 at physiological pH (Rothschild and Oratz, 1976). The isoelectric point of Alb is pH 4.9 (Schultze and Heremans, 1966). The amino acid sequence of human Alb is shown in Figure 1.

Alb is synthesized almost exclusively in the liver. Both rats and humans synthesize Alb at a rate of 0.4 mg/g liver/day. For humans, this translates into about 17 g Alb/day (Peters, 1975). A healthy 70 kg man has 350 g Alb, of which approximately 40% is in the plasma (Peters, 1975). However, during times of sickness, inadequate nutrition, or exposure to hepatotoxins, synthesis of Alb can be dramatically reduced (Rothschild et al., 1988). The concentration of Alb has long been used as an indicator of overall health, a notion aptly summarized by Peters, who noted that "happiness is a normal albumin level" (1975).
The basic functions of Alb have been known for over a century; they were first described by Ancell in 1839. Alb is required for the maintenance of osmotic pressure, as well as for the transport of a variety of substances, including bilirubin, fatty acids, metals, ions, hormones, and xenobiotics (Rothschild et al., 1988). Skipper and Tannenbaum (1990) postulated that the tendency of Alb to bind foreign compounds may increase the amount of adduct formation that occurs on Alb.

Methods of Albumin Isolation and Quantitation Used in the Measurement of Albumin Adducts

Alb-isolation procedures which can be applied to the analysis of Alb adducts must give reasonable yields in fairly high purity, must be applicable to plasma volumes of under 5 mL, should be rapid and easy enough to be applied to large numbers of samples, and most importantly, must not destroy the adduct during the isolation process.

Alb is usually isolated for analysis of adducts with one of three principle methods: selective precipitation involving ammonium sulfate, selective precipitation using ethanol, or affinity chromatography using a dye-linked matrix. Adducts which have been detected following precipitation with ammonium sulfate include 4-aminobiphenyl (Skipper et al., 1985), benzo[a]pyrene (Sherson et al., 1990), aflatoxin B₁ (Autrup et al., 1991, Sabbioni et al., 1990a, Wild et al., 1990), aflatoxin G₁ (Sabbioni and Wild, 1991), styrene (Rappaport et al., in press), trichloroethylene, trichloroacetate, and dichloroacetate (Stevens et al., 1992). Adducts
which have been detected following ethanol precipitation include benzene (Bechtold et al., 1992) and 4,4'-methylenebis(2-chloroaniline) (Cheever et al., 1991). Adducts which have been detected following affinity chromatography include monochloroacetic acid (Kaphalia et al., 1992), aflatoxin B₁ (Gan et al., 1988, Groopman et al., 1992, Sabbioni et al., 1987), 2-amino-3-methylimidazo[4,5-f]quinoline (Turesky et al. 1987), benzo[a]pyrene (Lee et al., 1988), and polycyclic aromatic hydrocarbons (Lee et al., 1991). The details of some of these procedures and their specific advantages and disadvantages will be described more fully in the discussion.

Considerations for choosing a method for quantitation of Alb include rapidity, reproducibility, applicability to large numbers of fairly small samples, accuracy, and lack of interfering compounds. For example, some of the colorimetric methods of protein quantitation are not accurate in the presence of ammonium ions while other methods are very time consuming. In general, a small aliquot of the total protein solution is taken for quantitation and then discarded. Consequently, prevention of damage to the protein or adduct is not a consideration as it is for Alb-isolation procedures.

Researchers involved in the analysis of Alb adducts use a variety of methods, including weight determination (Bechtold et al., 1992; Cheever et al., 1991; Skipper et al., 1985; Turesky et al. 1987), the biuret reaction (Autrup et al., 1991; Sabbioni et al., 1990b), the Lowry assay (Gan et al., 1988; Sherson et al., 1990), the Bradford method (Kaphalia et
al., 1992; Sabbioni et al., 1990a, 1991; Wild et al., 1990), the bromocresol green assay (Sabbioni et al., 1990b; Stevens et al., 1992), the bicinchoninic acid protein assay (BCA) (Groopman et al., 1992; Lee et al., 1988, 1991), and absorbance at 280 nm (Rappaport et al., in press).

The specifics of each of these isolation and quantitation procedures, along with a brief discussion of their advantages and disadvantages, are detailed in later sections.

Styrene Adducts of Albumin

Styrene (CAS No, 100-42-5) is an oily liquid used in the production of plastics and resins (Barale, 1991). Exposure to styrene occurs primarily in industrial settings by inhalation of styrene vapors, with 60-70% of the inhaled styrene being absorbed by humans (Bond, 1989). Styrene is mainly converted to the electrophilic species styrene-7,8-oxide (styrene oxide) by the cytochrome P450 monooxygenase system. Styrene oxide can then be further metabolized and excreted; 85% of the dose is eliminated as the urinary metabolites phenylglyoxylic acid and mandelic acid (Bond, 1989). However, styrene oxide can also react with biological macromolecules such as DNA, hemoglobin, and Alb to give the corresponding adducts.

Although the carcinogenicity of styrene has not been proven, a variety of genotoxic effects have been noted both in vitro and in vivo (Barale, 1991). In order to improve the validity of epidemiologic studies involving low-level exposures to styrene, a number of biomarkers of styrene exposure have been tested. These biomarkers include the DNA adducts,
hemoglobin adducts, and plasma protein adducts of styrene oxide (Brenner et al., 1991; Cantoreggi and Lutz, 1992; Nordqvist et al., 1985; Ting et al., 1990). As noted earlier, Alb adducts may be a more sensitive marker of exposure than hemoglobin adducts. Henderson et al. (1989) notes that studies which compare the levels of hemoglobin versus Alb adduct formation are needed. In order to undertake such a comparison for styrene adducts, the methodology for isolating and quantitating Alb first had to be developed.

This report describes the development of the isolation and quantitation procedure which has successfully been applied to the detection of styrene oxide-Alb adducts (Rappaport et al., in press). This research compared the formation of hemoglobin and Alb adducts when styrene oxide was reacted with human or rat blood in vitro and when either styrene or styrene oxide was administered to rats in vivo. When mixed with whole blood, styrene oxide was found to react rapidly with rat hemoglobin and Alb, moderately with human Alb, and slowly with human hemoglobin (with second order rate constants 72.4, 62.8, 31.7, 2.37 L/mol protein/hr, respectively). Administration of styrene oxide to rats resulted in much higher production of both Alb and hemoglobin adducts than styrene. Since this procedure has been shown to work both in vitro and in rats, it can now be applied to occupationally exposed humans. These results can then be compared to a number of other markers of exposure, including sister-chromatid exchange, micronuclei, DNA adducts, and exhaled styrene.
Review of Albumin-Isolation Methods

Since the isolation of Alb was first described by Hofmeister in 1890, hundreds of papers have been published describing either the selective isolation of Alb or the more general schemes to fractionate plasma into the constituent proteins. Stryker (1985) divides these fractionation procedures into three categories: methods based on interaction with physical parameters, with chemical precipitants, and with solid phases. Physical parameters which have been used to fractionate proteins include selective thermal denaturation, cryoprecipitation, ultracentrifugation, and electrophoresis. Chemical precipitants include organic solvents such as ethanol, acetone, methanol, and ether; neutral salts such as ammonium sulfate, sodium sulfate, magnesium sulfate, and phosphates; high molecular weight polymers such as polyethylene glycol; and other compounds such as zinc, polyphosphates, Rivanol, caprylic acid, trichloroacetic acid, and glycine. Techniques of plasma fractionation involving solid phases include affinity chromatography, ion exchange chromatography, ultrafiltration, and gel filtration. (reviewed in Stryker, 1985; Schultze and Heremans, 1966). Currently, most commercially available Alb is isolated using some modification of the ethanol precipitation procedures originally described by Cohn et al. in 1946 and 1950.

Methods which involve the isolation of Alb for use in the analysis of Alb adducts have employed only a small number of these isolation
techniques. The isolation procedures based on interaction with physical parameters have not been used because they either do not give high enough purity or are not applicable to a large number of samples. Chemical precipitants have received the most attention due to their ease of use and hence applicability to multiple analyses. Of the procedures involving solid phases, only affinity chromatography routinely yields high enough purity Alb to be applied to Alb adduct samples. Consequently, Alb is usually isolated for analysis of adducts using a variation on one of three principle methods: selective precipitation involving ammonium sulfate, selective precipitation using ethanol, or affinity chromatography using a dye-linked matrix. These three basic methodologies and the consequent removal of small molecules used in the isolation procedures are described in the following sections.

Salt Fractionation

The classic method used to precipitate proteins involves the use of high salt concentrations; methods incorporating this idea of "salting out" came into use over a century ago and continue to be important in plasma fractionation today. The ability of water to act as a solvent is based on its relatively ordered structure as dictated by its polarity and ability to form hydrogen bonds. Creighton (1993) states that "the solubility of a molecule in water depends on how much of the unfavorable aspects of creating a cavity in water are compensated by favorable interactions with the surrounding water molecules". However, high concentrations of other
molecules can alter the structure of water, causing its surface tension to increase and the solubility of nonpolar molecules to decrease. Multivalent anions such as sulfate are the most effective agents for salting out a protein (Creighton, 1993). Solubility of proteins depends upon the pH, ionic strength, temperature, dielectric constant, and concentration of protein in the solution. Maximum precipitation of a protein generally occurs near its isoelectric point (Pennell, 1960).

A selection of the techniques which have used ammonium sulfate to fractionate plasma are reviewed in Schultze and Heremans (1966). Originally, the globulins were defined as the proteins which precipitated when ammonium sulfate was at 50% saturation (2 M), while Alb was the fraction which remained in solution (Stryker, 1985). Current methods used for isolating Alb first bring the plasma to 50-65% ammonium sulfate saturation to remove the globulins, then lower the pH of the supernatant to pH 5, at which point Alb precipitates (based on Peters, 1962). The disadvantages of salt fractionation are that there is no clear separation between proteins (resulting in limited purity) and that the salt generally needs to be removed from the protein. However, salt fractionation is inexpensive, rapid, and readily applicable to a large number of samples.

Ethanol Fractionation

The use of ethanol to fractionate plasma became a generally accepted technique in 1946 when Cohn's group published methods that had been developed for large-scale isolation of blood components needed for World
War II (Cohn et al., 1946). By carefully controlling the ethanol concentration, pH, and temperature, a series of six fractions are obtained, with "fraction V" containing almost exclusively Alb. However, for small-scale systems or when Alb is the only component desired, Cohn's methods are far too complicated.

Other Alb isolation methods involving the use of ethanol were developed as early as 1932, when Race noticed that globulins and Alb could be separated by first adding trichloroacetic acid to plasma, followed by extracting the Alb from the precipitate with ethanol or acetone. This formed the basis of fractionation procedures based on Alb's solubility in acidified organic solvents. Variations on this method have been published by Debro et al. (1957), Kallee et al. (1957), Korner and Debro (1956), Levine (1954), Michael (1961), Rodkey (1965), and Schwert (1957). Investigators looking at Alb adducts who use ethanol in their isolation procedures tend to follow the version suggested by Fernandez et al. (1966) in which the globulins are first precipitated with acidic ethanol, and then the Alb is precipitated with sodium acetate in ethanol. Similar advantages and disadvantages apply to both salt-based precipitation procedures and ethanol-based precipitation procedures.

**Affinity Chromatography**

Purification of proteins using affinity chromatography involves the specific but reversible binding of the desired protein to a ligand linked to an insoluble gel matrix (Cuatrecasas, 1970). The protein of interest is desorbed from the column by changing the pH or ionic strength of the
elution buffer. A wide variety of both ligands and supports are currently
available which can be used to selectively isolate a number of different
proteins. In general the specific functional properties of a protein are
exploited to form complexes with the ligand. Examples of such complexes
include antibody-antigen, enzyme-substrate, and binding protein-
substrate.

Alb has been isolated using a number of different ligands such as
bromosulfophthalein-glutathione (Clark and Wong, 1979), alkyl succinic
acids (Aslam et al., 1976; Wichman and Andersson, 1974), and the dye
Cibacron Blue F3GA (initial work by Travis and Pannell, 1973; Travis et
al., 1976; Kelleher and Smith, 1979). Cibacron Blue F3GA is fairly
species-specific, with different binding affinities to different Albs.
Kelleher and Smith (1979) found a far lower affinity of rat, mouse, and
bovine Alb for Cibacron Blue than of human Alb under the same conditions
and Antoni et al. (1978) noted that the removal of Alb from different
species required the chromatographic conditions to be adjusted
appropriately. Supports that have been used include agarose (Sepharose,
Affi-Gel), dextrans (Sephadex), polyacrylamide, agarose-polyacrylamide
copolymers (Ultragel), cellulose, and glass (reviewed in Dean and Watson,
1979). The most common ligand-support matrix used to isolate Alb is
Cibacron Blue F3GA coupled to agarose, which is available under trade
names such as Affi-Gel Blue (Bio-Rad Labs, Richmond, CA) and Blue
Sepharose (Pharmacia, Uppsala, Sweden). Although affinity
chromatography is much more specific than precipitation procedures, the
technique can be more expensive and time-consuming (Bechtold, 1992). In addition, some concern has been raised about the stability of some adducts under the conditions used for affinity chromatography (Wild et al., 1990).

Removal of Small Molecules

Each of the isolation procedures outlined above yields salt, acid, or organic solvents in addition to Alb. These small molecules can interfere with protein quantitation or with the eventual use of the isolated protein. Investigators have dealt with this situation in a multitude of ways. Lyophilizing the Alb precipitate will remove organic solvents and washing the pellet can remove much of the acid or salt added. However, many of the procedures that attempt to remove these molecules involve the use of dialysis or gel filtration after redissolving the precipitate (if necessary). In dialysis, the solution is placed in a semipermeable tubing with pores that correspond to a specific molecular weight cutoff. Any molecule with a molecular weight less than the stated porosity comes to equilibrium with the surrounding water or buffer. Successive changes of the buffer result in eventual dilution of the small molecules to negligible concentrations. Gel filtration involves a matrix of porous beads. Molecules which cannot penetrate the pores are eluted in the void volume while those which can freely move in and out of the pores are eluted later. Dialysis tends to be time consuming but gel filtration can result in a substantial loss of protein.
Review of Albumin Quantitation Methods

Methods which have been used for measuring the amount of Alb which has been isolated include: gravimetric methods, specific gravity measurement, measurement of refractive index, nitrogen determination, spectrophotometric methods, atomic absorption, turbidimetric methods, and colorimetric methods such as the biuret method, the Lowry method, and methods based on binding to sulfobromophthalein, Sirius Supra red F3BA, Coomassie Brilliant Blue (Bradford method), trinitrobenzenesulphonic acid, methyl orange, bromocresol green, and bromocresol purple (reviewed in Darbre, 1986; Keyser, 1979). The Lowry method is the most widely used procedure today (Darbre, 1986).

Quantitation based on specific gravity, refractive index, nitrogen determination, atomic absorption, and turbidity have not been used in the analysis of Alb adducts primarily because these techniques are not applicable to routine determination of multiple samples. Most routine quantitation is based on spectrophotometric methods due to their relative speed and simplicity. Alb quantitation of samples used for adduct analysis has employed weight determination, absorbance at 280 nm, the colorimetric assays (biuret and Lowry), and dye-binding (Coomassie Brilliant Blue and bromocresol green). Only those methods which have been used in Alb adduct analysis will be described in this report.
Weight Determination

Quantifying the dry mass of protein is the most direct and straightforward method. Weight determination does not involve the use of standards, avoids the need for reagents, and does not require a difficult or tedious procedure. However, the method is non-specific and residual reagents or other proteins result in positive interferences. To assure the protein is free from reagents and other small molecules, the protein must be desalted or thoroughly washed after precipitation.

The Biuret Reaction

The biuret reaction was originally developed by Riegler in 1914. Since that time, a tremendous number of papers have been published which use modified versions of the biuret reaction. Examples of such applications include the work of Weichselbaum (1946) and Gornall (1949).

The procedure involves the addition of cupric sulfate to the protein in an alkaline solution of tartrate. The cupric ion forms a violet-red complex with the peptide linkage of the protein which can be monitored at 540 nm. All proteins and peptides longer than four residues are detected using the biuret reagent. The standard curve has a wide linear range, and there is only limited inter-protein variation. However, the sensitivity is very low (0.3 mg/mL) and ammonium ions tend to interfere with color formation (Schultze and Heremans, 1966). The biuret reaction forms the basis of commercially available total protein kits such as Sigma Diagnostics Kit 541-2.
The Lowry Assay

The Lowry assay (1951) is based on the biuret reaction; however, the addition of the Folin phenol reagent developed by Folin and Ciocalteu (1927) greatly improves the sensitivity of the reaction. The phosphomolybdic-tungstic mixed acids of the phenol reagent are reduced by the protein to give an absorption maximum at 720-750 nm. Tyrosine, tryptophan, histidine, asparagine, and the peptide backbone are known to be involved with the reaction. The sensitivity of the Lowry assay (0.2 ug/mL) is much greater than the biuret reaction (Darbre, 1986). However, the assay is less specific than the biuret reaction; consequently a number of non-protein substances are capable of interfering with the color formation (Schultze and Heremans, 1966). Lowry et al. (1951) noted that an ammonium sulfate concentration greater than 0.25% decreases the color development. In addition, the standard curve is non-linear. There is also wide variability of response for different proteins; therefore, the standards must have the same protein composition as the samples (Darbre, 1986).

The BCA Protein Assay

The BCA assay is a more recent modification of the biuret reaction. By combining the alkaline cupric sulfate reagent of the the biuret reaction with bicinchoninic acid, a purple solution forms (with an maximum absorbance at 562 nm) upon incubation with protein (Smith et al., 1985).
Cysteine, cystine, tryptophan, tyrosine, and the protein peptide bond have been shown to be responsible for color formation (Wiechelman et al., 1988).

After addition of the BCA reagent to the protein, the solution is incubated for 30 mins to 2 hours, depending upon the protein concentration and temperature of the reaction. The samples are then read in a spectrophotometer at 562 nm and compared to a standard curve. At lower temperatures, there is no clear endpoint of the reaction, so all samples need to be read within the same time frame. The BCA assay results in inter-protein variability similar to that seen in the Lowry assay. The sensitivity for the two assays is also similar (0.5 ug/mL) (Smith et al., 1985). Although much fewer reagents interfere with the BCA assay as compared to the Lowry assay, ammonium sulfate has been shown to reduce the amount of color development, resulting in an underestimation of protein concentration unless it is removed (Brown et al., 1989). The BCA protein assay is commercially available from Pierce as kit # 23225.

The Bradford Assay

The Bradford assay, developed by Bradford in 1976, makes use of the binding of the dye Coomassie Brilliant Blue G-250 to protein. The dye, which is dissolved in ethanol and phosphoric acid, forms a non-covalent complex with proteins when it is added to the sample. This binding causes the absorbance maximum to shift from 465 to 595 nm, so monitoring the solution at 595 nm allows protein concentrations to be determined from a standard curve. The dye solution is available commercially from Bio-Rad.
and Pierce.

This assay is four times more sensitive than the Lowry method, is not affected by ammonium sulfate, and is very simple. However, interference from acetic acid, non-linearity of response, and differences in binding among proteins have been reported (Darbre, 1986). Consequently, standards must consist of the same proteins as in the solution of interest. Pierce and Suelter (1977) noted that the reagent was stable for two weeks and the assay was rapid and sensitive. However, they go on to say that "if sufficiently concentrated protein solutions are available (minimum of 50 ug/mL), it is our viewpoint that measurements at 280 nm can be standardized in a similar manner and can be made with more rapidity and ease".

The Bromocresol Green Assay

Alb has a high affinity for the bivalent anion of the dye bromocresol green (Rodkey, 1965). After adding the dye solution to plasma, the absorbance at 628 nm can be monitored (Keyser, 1979). However, exact timing can be important; therefore, it has been recommended that absorbance measurements be taken 10 seconds after adding the bromocresol green reagent (Corcoran and Duran, 1977). This is the only method described in this report which allows the quantitation of Alb directly in plasma. However, for most studies involving protein adducts, the Alb does need to be isolated for later applications. Bromocresol green forms the basis for commercially available Alb determination kits such as
Absorbance at 280 nm

The aromatic residues tryptophan, tyrosine, and phenylalanine have a maximum absorbance at about 280 nm. As long as a protein contains at least one tryptophan, it can be detected by UV absorbance. As commented by Darbre (1986), "spectrophotometric methods have much to commend them. They are simple, rapid and non-destructive." The limit of detection of this method is about 0.1 mg protein/mL (Schultze and Heremans, 1966). This method requires no special reagents, and the protein is not destroyed in the process so it can be used in the later procedures. However, like the colorimetric reactions described above, the standards must have the same protein content as the samples or the results will be inaccurate. In addition, any turbidity or contamination with molecules that absorb at 280 nm will cause an overestimation of protein concentration.
Materials and Methods

Reagents

Whole rat blood was obtained from Sprague-Dawley rats by cardiac puncture into a heparinized syringe after the animals were anesthetized with methoxyfluorane. Plasma was separated from red blood cells by centrifugation at 800 x g for 10 minutes. Human serum, human Alb (Cohn fraction V, 96-99%), sodium acetate, and Sephadex G-25 (20-80 um) were purchased from Sigma (St. Louis, MO). Ammonium sulfate (99%), acetic acid (99.7+%), sodium phosphate, sodium thiocyanate, tris(hydroxymethyl)aminomethane were obtained from Aldrich (Milwaukee, WI). Sodium chloride, sodium azide, and Spectra/Por dialysis tubing (12-14,000 MW cutoff) were obtained from Fisher (Pittsburgh, PA). The Bradford protein assay reagent and Affi-Gel Blue were obtained from Bio-Rad (Richmond, CA). Blue Sepharose CL-6B was obtained from Pharmacia (Sweden). Water was purified with a Milli-Q system (Waters, Millipore Division, Bedford, MA). All SDS-PAGE reagents were obtained from the Dermatology Laboratories at Duke University Medical Center (Durham, NC).

Methods

Affinity Chromatography

A 20 x 1.5 cm glass column was filled with about 20 mL slurry of either Affi-Gel Blue or Blue Sepharose CL-6B (according to the manufacturers' specifications this was enough packing material to bind
about 100 mg Alb for Blue Sepharose and 200 mg Alb for Affi-Gel Blue). The column was attached to a Fluid Metering, Inc. Lab Pump and peaks were detected using a 254 nm Beckman Analytical Optical Unit coupled to an Omniscribe chart recorder. The column was equilibrated with two bed volumes of 0.02 M phosphate buffer (pH 7.1). Samples of plasma or Alb were applied to the column through an injection valve with a 7 mL sample loop. The protein which did not bind to the Cibacron blue was recorded as a single peak on the chart recorder (peak 1). When the detector returned to baseline, 1 - 4 M sodium chloride in 0.02 M phosphate buffer (pH 7.1) was used to elute the Alb, which also gave a single peak (peak 2). A typical example of a chromatogram is given in Figure 2. Both peaks were collected, desalted, and quantified using the techniques described later. The column was then reequilibrated with two bed volumes of phosphate buffer. If the peaks started to broaden, the column would be flushed with a regeneration buffer. Affi-Gel Blue was regenerated with two bed volumes of 1.5 M sodium thiocyanate in 0.02 M phosphate buffer. Blue Sepharose regeneration involved 4-5 cycles of alternating a high pH buffer (0.1 M Tris-HCl, 0.5 M sodium chloride, pH 8.5) with a low pH buffer (0.1 M sodium acetate, 0.5 M sodium chloride, pH 4.5). The column was either stored at 4°C or in a 0.01% sodium azide solution to prevent bacterial growth.

To optimize the operating conditions for the isolation of Alb from plasma, solutions of human Alb were used as a surrogate for plasma. The buffer flow rate (0.5 - 20 mL/min), volume (0.5 - 7 mL), pH (1 - 14),
concentration of Alb (5 - 40 mg/mL), and the salt concentration of the elution buffer (1 - 4 M) were all varied to determine the optimum conditions under which Alb bound to and was eluted from the column. By varying these parameters, it was hoped that a set of conditions could be determined (by looking at the heights of peaks 1 and 2) which resulted in the highest yield of Alb while minimizing sample run time and the volume in which the peaks eluted.

Ammonium-Sulfate Precipitation

A saturated solution of ammonium sulfate (about 4 M) was prepared by dissolving as much ammonium sulfate as possible in 1 L warm water (60°C) and allowing crystallization to occur as the solution cooled. To 1 - 5 mL of rat or human plasma the saturated ammonium sulfate solution was added dropwise to bring the final concentration of ammonium sulfate to 2.5 M (62% saturation) at room temperature. Immediate formation of a white precipitate was observed and the mixture was briefly vortexed and then centrifuged for 30 minutes at 3000 x g to form a solid pellet (mostly globulins). The supernatant was transferred to another centrifuge tube and the pH was lowered to the isoelectric point of Alb (pH 5) with acetic acid. The mixture was again vortexed and centrifuged to give a second pellet (mostly Alb). A flow chart of this procedure is given in Figure 4. Both precipitates were then individually redissolved in water, desalted, and quantified.

In order to optimize the purity and yield of the Alb, the following parameters were tested: speed of addition of ammonium sulfate, amount
of acetic acid (50 - 200 μL), temperature of sample (4 and 22°C), centrifuge time (10 - 60 minutes), and amount of sample used (0.5 - 5 mL). The weight of the precipitates could not be used to quantify the protein since they were contaminated with an unknown amount of ammonium sulfate or acetic acid. Protein concentration was used to estimate the effect of altering these different parameters. The final concentration of ammonium sulfate was not varied because overall protein content gave no indication of how well the Alb had been separated from the other plasma proteins. Consequently, a final ammonium sulfate concentration of 2.5 M was chosen by reviewing the available literature. The final procedure was verified by SDS-PAGE to confirm the location and purity of the Alb obtained from both human and rat plasma. The procedures used to determine protein concentration and to perform SDS-PAGE are described in the following sections.

**Removal of Small Molecules**

The Alb isolated by these above procedures contained salts (sodium phosphate, sodium chloride, ammonium sulfate), acetic acid, and other small molecules. These small molecules were removed by either gel filtration or dialysis. To prepare columns for gel filtration, Sephadex G-25 was first swollen in deionized water, then packed into 1 x 10 cm columns and washed 3 - 4 times with 5 - 10 mL deionized water by centrifugation at 800 x g for 4 minutes. Alb solutions were desalted by placing 2.5 mL on a column and centrifuging. The eluant contained the Alb while the small molecules were retained in the Sephadex.
In order to determine how many successive columns were required for "complete" removal of small molecules, Alb solutions were spiked with a small molecule (2-phenylethanol) to represent potential plasma contaminants which could interfere with the analysis of Alb adducts. The eluant was tested for both ammonium sulfate and 2-phenylethanol. Ammonium sulfate was detected by adding barium nitrate to the eluant and weighing the resulting barium sulfate precipitate. 2-phenylethanol was quantified using gas chromatography-mass spectrometry (Ting et al. 1990).

The dialysis tubing was prepared by briefly washing it in deionized water. The Alb solutions were then added to the dialysis tubing and dialyzed against 3 x 4 L deionized water at 4°C with constant stirring over the course of 30-50 hours. Ammonium sulfate and 2-phenylethanol were assayed as described above.

**Sodium Dodecyl Sulfate - Polyacrylamide Gel Electrophoresis**

Since SDS-PAGE allows the identification of the different components of a protein solution on the basis of the individual molecular weights, this technique was used to test the purity of the Alb obtained by ammonium-sulfate precipitation as described above. The procedure was performed according to the method of Laemmli (1970). The following protein solutions were tested: human serum, human Alb (fraction V from Sigma), and the two precipitates and the two supernatants from the ammonium
sulfate fractionation procedure. All solutions were diluted to approximately 1 mg/mL with deionized water as determined by absorbance at 280 nm or the Bradford protein assay. A high molecular weight standard (Bio-Rad) containing myosin (200,000), β-galactosidase (116,250), phosphorylase b (97,400), bovine serum Alb (66,200), and ovalbumin (42,699) was also run to identify the Alb band.

An 8% acrylamide gel was prepared by mixing 1.5 M Tris (pH 8.8), 0.2% SDS, 0.03% ammonium persulfate, and N,N,N',N'-tetramethylethylene diamine (Temed). This separating buffer was added to the gel apparatus and allowed to set under a layer of water-saturated butanol for at least two hours. After removing the water-saturated butanol, a stacking buffer containing 0.5 M Tris (pH 6.8), 0.1% SDS, 0.07% ammonium persulfate, and N,N,N',N'-tetramethylethylene diamine was then added and allowed to set. The samples (30 ug per well) were mixed with glycerol, 0.5 M Tris (pH 6.8), 2% SDS, and Bromophenol Blue and then placed on the gel. The running buffer contained 1.4% glycine, Tris, and 0.1% SDS. The gel was run at 30 amps until the tracking dye reached the bottom of the gel (4 hours). After removing the gel, it was stained with a solution containing 0.2% Coomassie Blue, 50% methanol, and 10% acetic acid. The gel was then destained overnight with a solution containing 25% acetic acid and 7.5% methanol. The gel was photographed and the negative scanned using a LKB 2202 Ultroscan Laser Densitometer. The relative band areas were determined by cutting out the peaks and weighing them. The precipitates and supernatants obtained from samples of rat plasma
were also assayed by SDS-PAGE to verify the ammonium-sulfate fractionation procedure was applicable to both humans and rats.

**Protein Quantitation**

Protein was quantified at 280 nm and 595 nm with a Spectronic 1201 spectrophotometer (Milton Roy Company, Rochester, NY). Protein concentration was determined using the Bradford protein assay by following the protocol accompanying the Bio-Rad protein assay kit. 5 mL assay reagent was added to 0.1 mL sample and mixed gently, and absorbance was read at 595 nm. A standard curve (0.2 - 2 mg/mL) was prepared in the same way as the samples using commercial human Alb. Alb concentration was determined using Beer's Law.

To determine the protein concentration using absorbance at 280 nm, the protein solutions were diluted with deionized water (if necessary) so that their absorbance was less than 1.5 when read at 280 nm. Concentrations were calculated based on a standard curve (0.2 - 2 mg/mL) using human Alb as described above.
Results

Albumin Isolation

Affinity Chromatography

When a sample was run through an column containing either Affi-Gel Blue or Blue Sephadex, two peaks were always obtained. The first peak (peak 1), containing protein that did not bind to the column, eluted with the 0.02 M phosphate buffer. The second peak (peak 2), containing the protein that did bind to the column, eluted in the sodium chloride-phosphate buffer. Theoretically, peak 1 contained no Alb and peak 2 consisted entirely of Alb. However, even when solutions of pure Alb were passed through the column, peak 1 still appeared. Figure 2 gives an example of a typical chromatogram obtained from 5 mL of 20 mg/mL Alb solution.

To determine if the column was being overloaded, volumes ranging from 0.5 to 7 mL and concentrations of Alb ranging from 5 to 40 mg/mL were tested. For both peak 1 and peak 2 a linear response was obtained when either sample volume or concentration was plotted versus peak height. If the column were being overloaded then peak 1 should have disappeared at low loadings. Both Affi-Gel Blue and Blue Sepharose responded similarly, although the Blue Sepharose gave smaller peak 1 areas. In addition, the pH of the sample solution had a significant effect on the relative areas of the peaks. At pH greater than 10, almost all the protein eluted in peak 1 with almost none in peak 2. At pH less than 2,
peak 1 split into two peaks, and at pH 5 the highest peak 2/peak 1 ratio was observed.

5 mL of plasma or Alb yielded peaks which eluted in 25-35 mL buffer. Since these solutions must be processed further (desalting, adduct analysis), the conditions were optimized to minimize both the run time per sample and the final volume of solution. Flow rates were varied from 1 to 15 mL/min without exceeding the recommended pressure of 15 psi. Concentration of sodium chloride in the Alb elution buffer was varied from 1 to 4 M. Both increasing the flow rate and salt concentration reduced the run time and the final volume of peak 2 to a certain degree. A standard flow rate of 5-7 mL/min was adopted and a salt concentration of 2.5 M for the elution buffer was found to be optimal. Under these conditions, the run time per sample was 10-15 mins, including the time required to reequilibrate the column with phosphate buffer.

Ammonium Sulfate Precipitation

After ammonium sulfate was added to a plasma sample, a fluffy white precipitate immediately formed (precipitate 1), leaving behind a slightly yellow solution (supernatant 1). When acetic acid was added to the supernatant another white precipitate formed (precipitate 2), leaving a much clearer solution (supernatant 2). Varying the speed of addition of ammonium sulfate suggested that this solution should be added in a dropwise fashion. Doing the precipitation at both 4°C and 22°C indicated that the warmer temperature resulted in less precipitation of both the
first and second precipitates. The time of centrifugation was found to be important for getting good separation between the precipitate and supernatant; centrifugation for 30 minutes at 3000 x g allowed the supernatant to be poured off without loss of the precipitate. Increasing the amount of acetic acid resulted in increases in the amount of protein in precipitate 2.

After optimizing the reaction conditions using protein quantitation assays, the specific protein content of the two precipitates and two supernatants was assayed using SDS-PAGE. Human plasma and Alb from Sigma (fraction V) were run for comparison. The results of this gel are shown in Figure 3. Based on a scan of the major bands, plasma appeared to contain 79% Alb and 16% globulins. These numbers were somewhat higher than expected since the multitude of smaller bands are almost non-detectable when compared with the Alb and globulin bands. Precipitate 1 contained a higher percentage of globulins (29%), although there was still a large proportion of the Alb (59%). Supernatant 1 consisted almost exclusively of Alb (93%), as did precipitate 2 (92%) and supernatant 2 (87%). There were no detectable globulins in any of these three fractions. In contrast, the Alb purchased from Sigma (fraction V) appeared to be only 70% pure. These results are given on the flow chart shown in Figure 4.

Rat plasma and its fractions gave similar results to the human plasma, as determined by SDS-PAGE.
Removal of Small Molecules

The results of the desalting efficiency of successive passes through the Sephadex columns were contradictory for 2-phenylethanol and ammonium sulfate (see Table 1). When testing removal of 2-phenylethanol, it was determined that one pass gave 98% removal, two passes gave 99.9% removal, and three passes reduced the level below our detection limit. In contrast, the removal of ammonium sulfate was much less efficient, with one pass resulting in 60% removal, two passes resulting in 86% removal, and three passes giving 96% removal.

Table 1

Efficiency of Sephadex Desalting:
Comparison of 2-phenylethanol and Ammonium Sulfate

<table>
<thead>
<tr>
<th># of passes</th>
<th>% removal of through column 2-phenylethanol</th>
<th>% removal of ammonium sulfate</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>98</td>
<td>60</td>
</tr>
<tr>
<td>2</td>
<td>99.9</td>
<td>86</td>
</tr>
<tr>
<td>3</td>
<td>not detectable</td>
<td>96</td>
</tr>
</tbody>
</table>

Other observations of the desalting columns indicated that the first time a sample was passed through a column, some compression of the gel occurred and the sample was diluted slightly (10-20% increase in volume). In addition, each time a protein solution was run through a column approximately 20% of the protein was lost.
The efficiency of dialysis was tested under the conditions described above. For a total sample volume of 40 mL, 3 x 4 L water changes is expected to result in virtually complete removal of small molecules (>99.9999%). No ammonium sulfate or 2-phenylethanol was detected following dialysis. Other observations concerning dialysis were that with actual plasma samples, a small amount of white precipitate formed inside the dialysis tubing. In addition, the protein solution was diluted (~10% increase in volume), and there was usually some loss of protein (5-10%) during the transfers to and from the dialysis tubing.

Protein Quantitation

The Bradford Protein Assay

Human Alb standards from 0.2 to 2 mg/mL were run using the Bradford assay to generate a standard curve with absorbances ranging from 0 to 1.4. This curve was only linear up to about 1 mg/mL, as shown in Figure 5. Saturated ammonium sulfate caused some interference at 595 nm, giving an absorbance of 0.09 (which roughly corresponds to 0.1 mg Alb/mL); acetic acid had a negligible effect. One difficulty with this assay was related to the stability of the reaction products. Thus, if a large number of samples need to be quantified at one time, the readings can vary considerably between the first and last determinations. In addition, there was a significant difference in response between proteins.
Absorbance at 280 nm

Human Alb standards from 0.2 to 2 mg/mL were measured at 280 nm to generate the standard curve shown in Figure 6. This curve was linear over the whole range tested; concentrations greater than this range (absorbance > ~1.3) were diluted first before measuring the absorbance. A saturated solution of ammonium sulfate had an absorbance at 280 nm of 0.06 (corresponding to ~0.1 mg Alb/mL) and the addition of acetic acid to the samples caused a slight increase in absorbance. There was almost no change in absorbance over time. However, different responses were again noted among proteins as seen with the Bradford assay. While a standard curve prepared from human Alb would give a slope of 0.6, a similar set of standards generated using isolated globulins resulted in a slope of 0.8-0.9.
Discussion

Albumin Isolation

Affinity Chromatography

Although a number of researchers have successfully isolated Alb using Cibacron blue affinity chromatography, a number of difficulties were encountered in this investigation. Of particular concern was the amount of Alb that did not bind to the column, regardless of the sample concentration or volume. Another disadvantage of the method, as employed by this study, was high salt concentration (2.5 M) and large volume (~30 mL) in which the Alb was eluted. There is also a greater chance of sample cross contamination than in procedures which do not reuse the column for subsequent samples. In addition, only 3 - 5 samples per hour could be run. Other investigators using affinity chromatography to isolate Alb have used somewhat different protocols, as outlined in Table 2.
Table 2
Affinity Chromatography Methods Used to Isolate Albumin for Use in the Analysis of Albumin Adducts

<table>
<thead>
<tr>
<th>Reference</th>
<th>Turesky, 87</th>
<th>Gan, 88</th>
<th>Groopman, 92</th>
<th>Kaphalia, 92</th>
</tr>
</thead>
<tbody>
<tr>
<td>Adduct</td>
<td>IQ</td>
<td>aflatoxin B1</td>
<td>aflatoxin B1</td>
<td>monochloroacetic acid</td>
</tr>
<tr>
<td>Species</td>
<td>rat</td>
<td>human</td>
<td>rat</td>
<td>rat</td>
</tr>
<tr>
<td>Plasma prep.</td>
<td>gel filtration</td>
<td>dilute x 2</td>
<td>dilute x 20</td>
<td>dialysis</td>
</tr>
<tr>
<td>Sample Vol</td>
<td>5 mL</td>
<td>2 mL</td>
<td>1 mL</td>
<td>not specified</td>
</tr>
<tr>
<td>Gel Type</td>
<td>Blue Sepharose</td>
<td>Blue Sepharose</td>
<td>Affinity-Filter</td>
<td>Affinity-Filter</td>
</tr>
<tr>
<td>Equil Buffer</td>
<td>10 mM Tris</td>
<td>50 mM Tris</td>
<td>0.05 M phosphate</td>
<td>0.05 M phosphate</td>
</tr>
<tr>
<td></td>
<td>0.2 M KCl</td>
<td>0.1 M KCl</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Elution Buffer</td>
<td>2 M KCl</td>
<td>1.5 M KCl</td>
<td>0.5 M phosphate</td>
<td>0.5 M NaCl</td>
</tr>
<tr>
<td>Concentration</td>
<td>ultrafiltration</td>
<td>ultrafiltration</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Desalting</td>
<td>dialysis</td>
<td>Sephadex G-25</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Other</td>
<td>pptn into</td>
<td>ultrafiltration</td>
<td>none</td>
<td>none</td>
</tr>
<tr>
<td></td>
<td>cold acetone</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Quantitation</td>
<td>weight</td>
<td>Lowry</td>
<td>bicinchoninic acid</td>
<td>Bradford</td>
</tr>
</tbody>
</table>

Sabbioni et al. (1987) and Lee and Santella (1988, 1991) also used Reactive Blue 2-Sepharose to isolate rat Alb, citing the procedure of Kelleher and Smith (1979). Kelleher and Smith examined the conditions necessary to purify rat Alb on a 24 x 9 cm column containing Cibacron blue-agarose. They showed that, by using 10 mM Tris as the starting
buffer, nearly 100% of both rat and human Alb bound to the column. With an NaCl concentration of 0.5 M, about 55-60% of the rat Alb was eluted, and at 2 M, 75% of the rat Alb was eluted. The report of 100% binding of human and rat Alb is not in agreement with the results obtained in this laboratory, since regardless of the conditions tried, there was always some Alb which did not bind to the affinity column. However, their stated salt concentrations needed for Alb desorption were consistent with the observations made in this laboratory that an NaCl concentration of 2.5 M resulted in maximum Alb yields.

Except for citing Kelleher and Smith, none of the authors reported Alb yields or purities for their application of affinity chromatography. However, some attempts have been made to reduce both the volume and concentration of salt of the final Alb solution. Turesky (1987) and Gan (1988) both concentrated the Alb solutions by ultrafiltration and then desalted them using either dialysis or gel filtration. Although high purity Alb was obtained in this manner, the procedures were time-consuming, and a substantial loss of Alb is expected. One other concern about affinity chromatography is the stability of the Alb adduct during the isolation procedure. Wild et al. (1990) mentioned that "by purifying the serum albumin by affinity chromatography with Cibacron blue (Gan, 1988) there is a major loss of aflatoxin-lysine". Based on the results obtained in this investigation and the descriptions from other researchers, it was decided that an effective and less time-consuming protocol could be developed using ammonium-sulfate fractionation
instead of affinity chromatography.

**Ammonium-Sulfate Precipitation**

Historically, it was found that at 50% ammonium sulfate saturation, the globulins precipitate, giving a crude separation from Alb. Keller and Block noted that at 50% saturation, the alpha, beta, and gamma-globulins precipitated, while Alb did not precipitate until 62-68% saturation (1960). Although some fraction of Alb is expected to precipitate, a concentration of 2.5 M ammonium sulfate (62-63% saturation) was chosen to allow for the highest possible purity of Alb to be obtained in a single step. The particular procedures used by others to isolate Alb for use in adduct work by using ammonium sulfate are outlined in Table 3.
Table 3
Ammonium-Sulfate Fractionation Procedures Used to Isolate Albumin for Use in the Analysis of Albumin Adducts

<table>
<thead>
<tr>
<th>Reference</th>
<th>Skipper, 85</th>
<th>Sabbioni, 90a</th>
<th>Wild, 90</th>
<th>Autrup, 91</th>
<th>Stevens, 92</th>
</tr>
</thead>
<tbody>
<tr>
<td>Adduct</td>
<td>4-aminobiphenyl aflatoxin B1</td>
<td>aflatoxin B1</td>
<td>aflatoxin B1</td>
<td>TRI, TCA, DCA</td>
<td></td>
</tr>
<tr>
<td>Species</td>
<td>rat</td>
<td>rat</td>
<td>human</td>
<td>rat &amp; mouse</td>
<td></td>
</tr>
<tr>
<td>Sample Vol</td>
<td>not specified</td>
<td>not specified</td>
<td>0.5 mL</td>
<td>0.25 mL</td>
<td>not specified</td>
</tr>
<tr>
<td>Final % Sat.</td>
<td>50%</td>
<td>56%</td>
<td>60% (0°C)</td>
<td>50%</td>
<td>50%</td>
</tr>
<tr>
<td>Centrifuge</td>
<td>9000 x g</td>
<td>not specified</td>
<td>9000 x g</td>
<td>4000 rpm</td>
<td>not specified</td>
</tr>
<tr>
<td></td>
<td>15 min</td>
<td></td>
<td>15 min</td>
<td>2 x 20 min</td>
<td></td>
</tr>
<tr>
<td>Alb precip.</td>
<td>1 N acetic acid</td>
<td>acetic acid</td>
<td>1M acetic acid</td>
<td>acetic acid</td>
<td>succinate + NaCl</td>
</tr>
<tr>
<td></td>
<td>until pH 5</td>
<td>until pH 5</td>
<td>0.1 mL, pH 5</td>
<td>not specified</td>
<td>until pH 4.8</td>
</tr>
<tr>
<td>Centrifuge</td>
<td>9000 x g</td>
<td>not specified</td>
<td>9000 x g</td>
<td>not specified</td>
<td>not specified</td>
</tr>
<tr>
<td></td>
<td>10 min</td>
<td></td>
<td>10 min</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Redissolve</td>
<td>50 mM phosphate</td>
<td>not specified</td>
<td>0.5 mL PBS</td>
<td>0.1M phosphate</td>
<td>not specified</td>
</tr>
<tr>
<td>Desalt</td>
<td>dialysis</td>
<td></td>
<td>dialysis</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Quantify</td>
<td>lyophilize &amp; weigh</td>
<td>Bradford</td>
<td>Bradford</td>
<td>biuret</td>
<td></td>
</tr>
<tr>
<td></td>
<td>bromocresol</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>green</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Yield</td>
<td>~50%</td>
<td></td>
<td>60-70%</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Purity</td>
<td>&gt; 95%</td>
<td></td>
<td>&gt; 95%</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Skipper et al. (1985) and Stevens et al. (1992) both used a
modification of the precipitation method originally described in 1962 by Peters. In an inappropriate application of this methodology, Sherson et al. (1990) used the pellet formed at 50% saturation to monitor benzo[a]pyrene adducts to serum proteins in general. They quantified the proteins using the protein-dependent Lowry method with bovine serum Alb as the standard. Since it is the globulins and not Alb which is expected to be in the pellet, the estimate of protein concentration obtained will be inaccurate.

Although most of these researchers couple the removal of the globulins with precipitation of the Alb at pH 5, the results of this investigation indicate that this second precipitation step is unnecessary if the globulin precipitation is carried out with an ammonium sulfate concentration of 2.5 M (~62% saturation). The protein gel (Figure 3) clearly shows that the solution remaining after the globulins are removed (supernatant 1) is almost exclusively Alb (~93%). The precipitate which forms at pH 5 (precipitate 2) is no more pure than the supernatant, and only results in the loss of more Alb, as indicated by the amount of Alb left in supernatant 2. Precipitate 1 contains an appreciable amount of Alb (~59%); however, by accepting a low recovery and a purity of 93%, Alb can be isolated in a single precipitation step. For the intended application of the isolated Alb in this laboratory, there is enough sample to justify losing a large fraction of it in order to obtain Alb in fairly high purity with a rapid, simple procedure.

The removal of the excess ammonium sulfate can be accomplished by
either gel filtration or dialysis. This research showed that although the small molecule, 2-phenylethanol, was 99.9% removed after passing the solution through two desalting columns, only 86% of the ammonium sulfate was removed at the same time. Since dialysis performed as described should result in virtually complete removal of both 2-phenylethanol and ammonium sulfate, and because much less Alb is lost in the process, this laboratory has chosen dialysis as the preferred method for desalting the Alb solutions.

Protein Quantitation

In order to quantify the Alb isolated by precipitation with ammonium sulfate, both the Bradford protein assay and absorbance at 280 nm were attempted. The biuret assay, with its low sensitivity, and the Lowry assay, with its tendency to cross react with a number of reagents, were not used in this investigation. Both the Bradford protein assay and absorbance at 280 nm are protein specific, which means the standards must be similar in content to the samples being tested. The use of Sigma Alb (fraction V) as a standard is valid for either procedure, since the constituent proteins of both fraction V and the Alb isolated using ammonium-sulfate precipitation are very similar (Figure 3). However, this research showed that the standard curve obtained with the Bradford protein assay is not linear over the range tested, and the absorbance readings had a tendency to drift with time. Although the Bradford assay is more sensitive than absorbance at 280 nm, the Alb isolated from
plasma using ammonium sulfate has been found to be sufficiently concentrated to be measured at 280 nm. Consequently, absorbance at 280 nm was the method selected by this laboratory to quantitate Alb.

Summary of Procedure for the Isolation and Quantitation of Albumin

After separating the plasma from red blood cells by centrifugation, a 1 - 5 mL human or rat plasma sample is brought to room temperature. A saturated solution of ammonium sulfate is added dropwise to the plasma until the final concentration reaches 2.5 M (~62% saturation). The solution is vortexed briefly, then centrifuged at 3000 x g for 30 minutes. The supernatant is transferred to dialysis tubing and stirred at 4°C for 30-50 hours with 3 x 4 L changes of deionized water. The absorbance at 280 nm of an aliquot of dialyzed Alb solution is recorded. The Alb concentration is calculated from standards prepared from human Alb and read in the same manner. A flow chart summarizing this procedure is given in Figure 7.
Conclusion

A method for the isolation and quantitation of Alb has been developed on the basis of descriptions in the literature and the results obtained in this laboratory. This procedure gives fairly high purity Alb in reasonable yield using both rat and human plasma. The methodology is straightforward and not very time-intensive. In addition, use of ammonium sulfate should not cause accidental cleavage of most adducts as has been reported for affinity chromatography. Moreover, the avoidance of the acetic acid precipitation of Alb allows the application of this method to protein adducts which are acid-labile. The quantitation of Alb by absorbance at 280 nm is easy and not time-dependent, and the use of Alb purchased from Sigma as a standard was shown to be valid. This procedure has been successfully used by this laboratory to isolate Alb for the detection of styrene oxide-Alb adducts (Rappaport et al., in press).
Figure 1

Amino Acid Sequence of Human Albumin

(Peters, 1975, p. 143)

Fig. 4. Amino acid sequence of human albumin displayed, like Fig. 3, in a model showing the proposed alignment of cystine bonds. Residues assigned by analogy with bovine albumin are shown in lower case letters. From Behrens et al. (1975). Reprinted from Fed. Proc. 34:591 (abstr. 2106), 1975.
Figure 2
Typical Affinity Chromatography Trace
(5 mL of 20 mg/mL Human Albumin Solution injected)
Figure 3

Protein Gel (SDS-PAGE)

Lane 1: High MW Standard
Lane 2: Sigma Human Albumin, Fraction V
Lane 3: Human Plasma
Lane 4: Precipitate 1
Lane 5: Supernatant 1
Lane 6: Precipitate 2
Lane 7: Supernatant 2
Figure 4

Isolation of Albumin from Plasma: 
Protein Content of Each Fraction 
(results of SDS-PAGE)

plasma
(79% Alb) 
(16% globulin)

ammonium sulfate

precipitate 1 
(59% Alb) 
(29% globulin)

supernatant 1
(93% Alb) 
(globulin not detectable)

pH 5

precipitate 2 
(92% Alb) 
(globulin not detectable)

supernatant 2 
(87% Alb) 
(globulin not detectable)
Figure 5
Protein Quantitation Using the Bradford Protein Assay

Standard Curve: [albumin] vs Abs @ 595 nm

\[ y = 0.0387 + 1.3645x - 0.3568x^2 \]

Figure 6
Protein Quantitation Using Absorbance at 280 nm

Standard Curve: [albumin] vs Abs @ 280 nm

\[ y = 0.0036 + 0.5921x \]
Isolation and Quantitation of Albumin

whole blood
  | centrifuge
  
red blood cells | plasma
  | ammonium sulfate
  |
  supernatant | globulins
    | dialysis
    |
albumin (~93% pure)
  | absorbance at 280 nm
    | adduct analysis
References


Race, J. (1932) The determination of blood-proteins by acid-acetone,


