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Note

Evaluation of the relative efficacy of various techniques for deproteinizing plasma samples prior to high-performance liquid chromatographic analysis

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The methods used for treating biological samples prior to their introduction into a high-performance liquid chromatographic (HPLC) system generally fall into one of two categories — extraction or direct injection. In the extraction method the compound of interest is removed from the biological matrix (plasma, serum, urine, etc.) using suitable solvent and pH conditions, which selectively extract the desired components and leave behind unwanted materials. The solvent is then removed by gentle evaporation and the dried residue reconstituted in a small volume of the elution solvent (or one quite similar to it) for injection on to the HPLC column.

The direct-injection technique is by far the simplest and most rapid of the two methods. In this procedure the biological sample may be injected directly on to the top of the HPLC column [1]. However, a number of reports have indicated that this results in a rapid increase in back-pressure and a deterioration of column performance [2—5], presumably due to the precipitation of plasma proteins as a result of their contact with the organic solvents and buffer salts commonly utilized in mobile phases [4]. To alleviate this problem, a number of sample preparation techniques have been described for removing proteins prior to injection of the sample. These include the use of precolumns [6], ultrafiltration devices [7, 8], and various protein precipitants such as organic solvents [9—11] and ionic salts [12—14].

Only one report dealing specifically with sample preparation procedures for the direct-injection HPLC technique has appeared to date [15]. In that study, six different methods of deproteinizing plasma were evaluated using the biuret assay to assess their efficiency. In the present report we describe a number of other potentially useful methods of protein removal, using the much more sensitive Lowry [16] method of protein determination to evaluate

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their efficacy. This latter point is important since even small amounts of residual protein will build up rapidly at the head of a HPLC column under conditions of high sample throughput, thereby necessitating more frequent column regeneration or replacement.

EXPERIMENTAL

Precipitating agents

The following precipitating agents were used: acetone, B.P.C. (Evans Medical Co., Liverpool, Great Britain); acetonitrile, AnalaR (BDH, Poole, Great Britain); ethanol, AnalaR (James Burroughs, London, Great Britain); methanol, AnalaR (James Burroughs); ammonium sulfate [$(NH_4)_2SO_4$] (saturated solution), AnalaR (Hopkin and Williams, Chadwell Heath, Great Britain); trichloroacetic acid (TCA), 10% (w/v) AnalaR (BDH); perchloric acid ($HClO_4$), 6% (w/v), AnalaR (BDH); metaphosphoric acid (HPO_3), glacial, sticks, 5% (w/v) (Fisons, Loughborough, Great Britain); sodium tungstate dihydrate ($Na_2WO_4 \cdot 2H_2O$), 10% (w/v), plus 0.67 *N* sulfuric acid, both AnalaR (BDH); zinc sulfate heptahydrate ($ZnSO_4 \cdot 7H_2O$), 10% (w/v), plus 0.5 *N* sodium hydroxide, both AnalaR (BDH); zinc sulfate heptahydrate ($ZnSO_4 \cdot 7H_2O$), 5% (w/v), plus 0.3 *N* barium hydroxide, both AnalaR (BDH); copper sulfate pentahydrate ($CuSO_4 \cdot 5H_2O$), 5% (w/v), plus sodium tungstate dihydrate, 6% (w/v), both analaR (BDH).

Plasma samples

All protein precipitation studies were performed using a single lot of pooled human plasma collected over lithium heparin from two healthy human volunteers. The total protein content of this pooled plasma sample was 89.3 g per 100 ml. All sample preparation techniques for a given precipitant were performed on the same day and the supernatants (or ultrafiltrates) assayed later that day.

Protein removal procedures

Precipitation methods. A series of 5-ml glass test-tubes, each containing 0.5 ml of plasma and the following quantities of precipitant — 0.1, 0.2, 0.3, 0.4, 0.5, 0.75, 1.0, 1.5 and 2.0 ml — were prepared in triplicate. In the case of those precipitants consisting of two ingredients, equal volumes of each component were added to provide the volumes of precipitant desired. The tubes were then rotated on a Vortex mixer for 30 sec, allowed to stand at room temperature for 15 min, and centrifuged at 1650 *g* for 15 min.

Each supernatant was collected, its pH measured (pH Meter Type PHM 51, Radiometer, Copenhagen, Denmark), and 0.1-ml aliquots were taken and assayed for protein content by the Lowry method [16] using suitable controls.

Ultrafiltration. To assess the efficacy of ultrafiltration in removing protein from plasma, triplicate 0.5-ml aliquots of pooled plasma were placed in Centriflo membrane cones (Type CF-25 and CF-50, Amicon Corp., Lexington, MA, U.S.A.) and centrifuged at 720 *g* for 30 min. This procedure yielded approximately 0.2 ml of ultrafiltrate from each cone. The individual ultrafiltrates were then assayed using the Lowry method [16].

RESULTS AND DISCUSSION

The efficacy of the various precipitants in removing the protein from plasma samples is shown in Table I. The data indicate that only very small quantities of 10% (w/v) trichloroacetic acid and 6% (w/v) perchloric acid are needed to remove > 98% of the protein present in plasma. At a 1 : 1 (v/v) ratio of precipitant to plasma only methanol and saturated ammonium sulfate solution failed to remove > 90% of the plasma protein. In spite of their relatively low efficacy in removing plasma proteins, the four organic solvents (methanol, ethanol, acetone, and acetonitrile) have been very popular as precipitants in the direct-injection HPLC technique because of their widespread use as mobile phase components. Their relative order of effectiveness in precipitating protein is acetonitrile > acetone > ethanol > methanol, which is approximately inversely related to their polarity.

Ammonium sulfate is a classical protein precipitant which functions as a result of its ability to compete sucessfully with protein molecules for the available water in the system. While the efficacy of this precipitant could probably be improved by controlling the pH so that the plasma proteins are at or near their isoelectric points, this procedure does not appear to be very popular because other, more efficacious precipitants are available.

The remainder of the precipitation methods examined here are more commonly used and depend upon the formation of insoluble salts. The best of these precipitants appear to be the four anionic types — trichloroacetic, perchloric, tungstic, and metaphosphoric acids. They are believed to function by forming insoluble salts with the positively charged amino groups of the protein molecules at a pH below their isoelectric point. The control of pH is especially important in the case of tungstic and metaphosphoric acids, as pointed out by Berkman et al. [17] and Briggs [18]. This is verified by the data in Table I.

The remaining three precipitants tested consisted of the heavy metal cations zinc and copper. It was once believed that these cations formed insoluble salts with protein molecules due to their interaction with the negatively charged carboxyl groups on the protein at pH values above the isoelectric point. However, the exact mechanism of this insoluble-salt formation is still unclear. These agents were proposed originally by Somogyi [19—21] and have not been widely used in conjunction with HPLC techniques due to the greater efficacy and ease of use of the anionic precipitants and the organic solvents mentioned earlier.

The CF-25 and CF-50 ultrafiltration cones were found to remove 99.8 ± 0.06% and 99.5 ± 0.31% (mean ± S.D.), respectively, of the plasma protein. These results indicate that either type of membrane (molecular weight cut-off 25,000 or 50,000) provides nearly complete removal of plasma proteins.

The method is relatively simple and offers a number of advantages over the protein-precipitation procedures that were outlined by Farese and Mager [22]. However, it should be realized that analysis of an ultrafiltrate will provide a measure of non-protein-bound drug, as opposed to total drug. In addition, separate experiments must be carried out to determine, and correct for, any binding of the drug (and the internal standard if added before ultra-

TABLE I

THE RELATIVE EFFICACY OF VARIOUS PROTEIN PRECIPITANTS

The results are expressed as the percentage plasma proteins precipitated and represent the mean of triplicate determinations.

Precipitant	pH of supernatant*	Volume of precipitant added per volume of plasma								
		0.2	0.4	0.6	0.8	1.0	1.5	2.0	3.0	4.0
10% (w/v) TCA	1.4—2.0 (3.1)	99.7	99.3	99.6	99.5	99.5	99.7	99.8	99.8	99.8
6% (w/v) $HClO_4$	<1.5 (2.0)	35.4	98.3	98.9	99.1	99.1	99.2	99.1	99.1	99.0
Tungstate—H_2SO_4	2.2—3.9 (7.0, 6.5)	3.3	35.4	98.6	99.7	99.7	99.9	99.8	99.9	100.0
5% (w/v) HPO_3	1.6—2.7 (4.8)	39.8	95.7	98.1	98.3	98.3	98.5	98.4	98.2	98.1
$CuSO_4$—Na_2WO_4	5.7—7.3 (8.0)	36.5	56.1	78.1	87.1	97.5	99.8	99.9	100.0	100.0
$ZnSO_4$—NaOH	6.5—7.5 (8.0)	41.1	91.5	93.0	92.7	94.2	97.1	99.3	98.8	99.6
$ZnSO_4$—$Ba(OH)_2$	6.6—8.3 (8.7)	45.6	80.7	93.5	89.2	93.3	97.0	99.3	99.6	99.8
Acetonitrile	8.5—9.5	13.4	14.8	45.8	88.1	97.2	99.4	99.7	99.8	99.8
Acetone	9 —10	1.5	7.4	33.6	71.0	96.2	99.1	99.4	99.2	99.1
Ethanol	9 —10	10.1	11.2	41.7	74.8	91.4	96.3	98.3	99.1	99.3
Methanol	8.5—9.5	17.6	17.4	32.2	49.3	73.4	97.9	98.7	98.9	99.2
Saturated $(NH_4)_2SO_4$	7.0—7.7	21.3	24.0	41.0	47.4	53.4	73.2	98.3	—**	—**

*Values in parentheses represent the pH of supernatants where the volume of precipitant per volume of plasma was 0.2, and 0.4 in the case of Tungstate —H_2SO_4.

**Cloudiness present in these samples precluded their accurate assay.

filtration) to the membrane. Finally, it may be necessary to consider the effect of the volume of ultrafiltrate collected on the concentration of drug in the ultrafiltrate to ensure that the binding equilibrium is not disturbed by the filtration process [23, 24], although recent reports indicate that binding equilibria are not perturbed by ultrafiltration [25, 26].

The decision as to which method to choose for a given analytical application must ultimately be determined by an assessment of such factors as the stability, recovery, membrane binding, etc., of the compound of interest and the precision of the assay. In fact, combinations of some of these methods, such as the use of mixed organic solvents [27], or organic solvents plus inorganic salts to help salt out the proteins [28, 29], may prove most suitable. Other seemingly unimportant factors such as the relative centrifugal force used to pack the precipitated proteins [30], and the temperature of the precipitants [31, 32], may prove to be the critical factors in determining the acceptability of a given preparative technique.

It is hoped that the data presented here will assist the analyst in optimizing the sensitivity of an assay and will help to resolve much of the dialog (usually undocumented) that has appeared in the literature recently [5, 11, 33–36] regarding the need for specific ratios of precipitant to plasma to achieve "complete" removal of protein.

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