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Optimization of protein precipitation based upon effectiveness of protein removal and ionization effect in liquid chromatography–tandem mass spectrometry

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Abstract

Four categories of protein precipitation techniques (organic solvent, acid, salt and metal ion) were tested in plasma using spectrophotometry to assess protein removal efficiency across a range of volumes, species and lots. Acetonitrile, trichloroacetic acid (TCA) and zinc sulfate were found to be optimal at removing protein in their categories (>96, 92 and 91% protein precipitation efficiency at a 2:1 ratio of precipitant to plasma, respectively). A post-column infusion LC–MS/MS system was used to assess ionization effect of a protein-bound drug caused by the endogenous components remaining after using various protein precipitants. The extent of ionization effect varied with mobile phase (−20 to 93%), protein precipitant (0.3–86%), but only slightly with species (86–93%). The optimal bioanalytical methodologies for removal of plasma proteins and minimal ionization effect for the probe molecule in positive ion turboionspray LC–MS/MS involve the use of TCA for precipitation with mobile phases consisting of either pure organic solvents (methanol:water or acetonitrile:water) or precipitation with all of the mass spectrometer compatible precipitants evaluated with a methanol:aqueous 0.1% formic acid mobile phase.

Keywords: Proteins

1. Introduction

In the pharmaceutical industry LC–MS/MS has become the method of choice for drug analysis in biological matrices. As a result, sample preparation and ionization effect issues have come to the forefront. Protein precipitation is commonly used for fast sample clean-up and disrupting protein–drug binding. This study evaluated various protein precipitants by examining their effectiveness at protein removal spectrophotometrically and the extent of ionization effect in positive ion turboionspray LC–MS/MS with common reversed-phase LC solvent mixtures.

The differences between the plasma of various species, used in early phase toxicology studies, and lot-to-lot variability in the efficiency of protein precipitants was studied. This is of particular interest

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as a single bioanalytical method is often developed for several animal species (dog, rat, mouse and human).

In order to quantify drug in a plasma sample, it is often necessary to disrupt the protein–drug binding so that the total amount of drug can be extracted for analysis. Precipitation is useful as it can be used to denature the protein, destroying its drug binding ability depending on the binding mechanism [1]. Not only is protein precipitation a very simple and fast procedure, it can also be used as a precursor to other methods.

The purpose of this study is to evaluate the various protein precipitants for bioanalytical LC–MS/MS. Inter-species and lot-to-lot variability for absolute protein precipitated will be addressed with an array of different precipitants, together with the ionization effect for LC–MS/MS analysis with standard reversed-phase LC solvent systems. The probe molecule chosen possesses both carboxylic acid and imine functionalities and is conventionally analyzed in positive ion turboionspray mode for optimum sensitivity (compound A, shown in Fig. 1).

The spectrophotometric assay monitoring at a wavelength of 280 nm is a measure of aromatic amino acid content. The assay was used in this study to determine protein concentrations in the supernatant of protein-precipitated plasma for several reasons. The spectrophotometric assay is simple, convenient, rapid (5–10 min), sensitive and requires a small sample size. Most buffers and ammonium sulphate do not interfere with the assay, and absorption is related to protein concentration [2]. Nucleic acid interferences are corrected for using the equation proposed by Layne [3]. Layne's formula relates protein concentration in a solution containing nucleic acid to absorption: protein concentration (mg/ml)= $1.55\ A_{280} - 0.76\ A_{260}$. The formula for nucleic acid correction is explained with the derivation of Layne's equation [4]. This study utilized the spectrophotometric assay monitoring at 280 nm with the correction for nucleic acids.

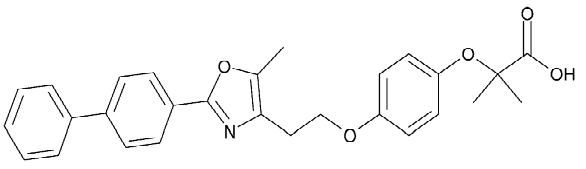

Fig. 1. Structure of compound A.

Research on protein precipitation is quite extensive. Many researchers have used protein precipitation in their analysis of drugs in serum and plasma. Often the precipitant used and the optimal volumes of precipitant to biological matrix differ between researchers, even for analysis of the same or similar drugs [5–14]. Comparison of protein precipitants in various animal species, which are typically used in toxicological and early phase drug studies, and variation in different lots of plasma has not previously been shown.

The different protein precipitation techniques (organic solvent, acid, salt and metal ion) have different modes of protein precipitation [15–17]. Protein solubility results from polar interactions with the aqueous solvent, ionic interactions with salts and repulsive electrostatic forces between like charged molecules. At the isoelectric point (p*I*), there is no net charge on a protein, and consequently the protein has minimum solubility in aqueous solvent. Above the p*I*, a protein has a net negative charge while below its p*I*, it has a net positive charge.

Precipitants exert specific effects on proteins to facilitate their precipitation from solution. Organic solvent precipitants lower the dielectric constant of the plasma protein solution, which increases the attraction between charged molecules and facilitates electrostatic protein interactions. The organic solvent also displaces the ordered water molecules around the hydrophobic regions on the protein surface. Hydrophobic interactions between proteins are minimized as a result of the surrounding organic solvent, while electrostatic interactions become predominant and lead to protein aggregation. Acidic reagents form insoluble salts with the positively charged amino groups of the protein molecules at pHs below their p*I*. Proteins are precipitated from solutions with high salt concentrations as the salt ions become hydrated and the available water molecules decrease, drawing the water away from the protein hydrophobic surface regions which in turn results in aggregation of protein molecules via protein–protein hydrophobic interactions. The binding of positively charged metal ions reduces protein solubility by changing its isoelectric point (p*I*). Metal ions are in competition with solution protons for the coordination binding

sites on the exposed amino acids. The stronger binding metal ions displace the protons from the binding sites, resulting in a lowering of the solution pH. The combination of changing the protein's p*I* and lowering the pH generally succeeds in precipitation of proteins.

Ionization effect in LC–MS/MS should be considered [18], whether direct injection after protein precipitation or an extraction method is employed. Ionization effect is one of the most common reasons for assay failure. Miller-Stein et al. [19] developed a post-column infusion method that allows identification of chromatographic regions of ionization effect. A steady-state signal of analyte infused into the mobile phase is compared to the variation in signal that occurs after injection of a plasma blank extract. The study described here used this procedure to assess the degree to which each protein precipitant removed endogenous plasma components causing suppression or enhancement of analyte signal. The variations of ionization effects with precipitant, mobile phase and species were measured by comparison of the difference in intensity of the signal of a water blank and the supernatant of protein precipitated plasma samples throughout the region of ionization effect observed using the chosen LC mobile phase and precipitant combination.

2. Experimental

2.1. Materials

Dog, rat and mouse plasma were acquired from Harlan Bioproducts for Science (Indianapolis, IN, USA) and human plasma from Biological Specialty (Colmar, PA, USA). The anticoagulant used for all plasma was sodium heparin. The following chemicals were used in the preparation of the protein precipitants and/or mobile phases: acetonitrile (Omnisolv, EM Science, Gibbstown, NJ, USA), aluminum chloride (99%, Aldrich, Milwaukee, WI, USA), ammonium sulfate (A.C.S. reagent, Aldrich, Milwaukee, WI, USA), formic acid (AnalaR, EM Science, Gibbstown, NJ, USA), formic acid–ammonium salt (Sigma, St. Louis, MO, USA), methanol (high purity, Burdick and Jackson, Muskegon, MI, USA), *m*-phosphoric acid (A.C.S. reagent, Aldrich, Milwaukee, WI, USA), reagent alcohol (HPLC, Fisher, Nepean, Ont., Canada), sodium hydroxide (1 N, certified, Fisher, Nepean, Ont., Canada), trichloroacetic acid (Aldrich, Milwaukee, WI, USA) and zinc sulfate (A.C.S. reagent, Aldrich, Milwaukee, WI, USA). Pre-diluted bovine serum albumin (BSA) standards were obtained from Pierce Chemical (Rockford, IL, USA) and calf thymus deoxyribonucleic acid (DNA sodium salt) from Sigma, St. Louis, MO, USA. DNA standards were prepared in disodium hydrogen phosphate solution (Sigma, St. Louis, MO, USA).

2.2. Protein precipitation procedure

The following protein precipitant solutions were prepared: aqueous ammonium sulfate (saturated at room temperature), aqueous aluminum chloride (5%, w/v), aqueous *m*-phosphoric acid (5%, w/v), aqueous trichloroacetic acid (TCA) (10%, w/v), (1:1) aqueous zinc sulfate heptahydrate (10%, w/v):0.5 N sodium hydroxide, acetonitrile, ethanol and methanol. Each precipitant was added to dog, rat, mouse and human plasma in volume ratios of 0.5:1, 1:1, 1.5:1, 2:1, 3:1 and 4:1 in triplicate and to four human plasma lots in a ratio of 2:1 for the spectrophotometric assay. Solutions were vortexed for 20 s, left to stand for 20 min (acid precipitated solutions were refrigerated at ~4 °C) and centrifuged for 10 min at 3000 rpm (rotor arm length: 12 cm). The absorbance of the supernatant was measured on a single beam diode array spectrophotometer relative to appropriate blanks at 280 nm. Plasma protein concentration was determined and compared to that of non-precipitated plasma (total) where precipitation efficiency = [(total plasma protein–protein remaining in supernatant)/total plasma protein] × 100.

2.3. Protein concentration determination

A total of seven BSA protein standards were prepared using sequential dilution from 1.5 to 0.025 mg/ml and were assayed at 280 nm. Nucleic acid interference was investigated by determining the A_{280}/A_{260} ratio of the plasma. A total of eight calf thymus DNA standards (2–100 μg/ml) were prepared using serial dilution in 1 m*M* disodium hydrogen phosphate solution and assayed at 280 and 260

nm. The protein precipitants were added to the DNA standards in a 2:1 ratio following the procedure for protein precipitation. The reduction in absorbance at 260 nm was examined. For the plasma supernatants, the equation: protein (mg/ml) = 1.55 A_{280} − 0.76A_{260} [3] was used to determine protein concentration. The validity of the assumptions that the A_{280}/A_{260} ratio for pure proteins = 1.75 and that the A_{260}/A_{280} ratio for pure nucleic acids = 1.8 (DNA) − 2 (RNA) was verified using the protein and DNA standards.

2.4. Measurement of ionization effect in LC–MS/MS

A tandem mass spectrometer (PE Sciex Model API III plus™, PE Sciex, Thornhill, Ont., Canada) equipped with a turboionspray interface was set up with a post-column infusion system (Harvard Apparatus infusion pump, Holliston, MA, USA). A 1-μg/ml solution of compound A was constantly infused post-column at 10 μl/min. The mass transition for compound A was *m*/*z* 458.0 to 262.1 using a positive ion SRM detection mode. The HPLC pump used was a Shimadzu SIL-10AD with a Shimadzu SIL-10A_{xl} autoinjector (Shimadzu, Columbia, MD, USA).

Five mobile phase solvent systems (0.5-ml/min flow rate) were used: 50:50 methanol:water, 70:30 methanol:water, 50:50 acetonitrile:water, 50:50 methanol:0.1% formic acid, and 50:50 methanol:10 m*M* ammonium formate. A Waters Symmetry Shield, 50×2.1 mm, 3.5 μm RP8 HPLC column was employed (Waters, Milford, MA, USA). Protein was precipitated in plasma using each protein precipitant in a 2:1 volume ratio as per the procedure described above with the exception that the samples were centrifuged at 10 000 rpm for 10 min (rotor arm length: 5 cm). Blank samples were prepared using water instead of plasma. Injections (25 μl) of each blank and supernatant were made in duplicate. The extent of ionization effect was calculated throughout the complete region of effect using the formula: ionization effect (%) = [average intensity of precipitated plasma supernatant/average intensity of water supernatant] × 100. The retention time of the analyte using the precipitant and mobile phase solvent mixtures was determined.

3. Results and discussion

The BSA standard curve at 280 nm was found to be linear with a correlation coefficient of 0.999 and yielded an extinction coefficient for BSA of 0.67 cm^{-1} ml/mg, which is comparable to the literature value of 0.7 cm^{-1} ml/mg [4]. An example standard curve is shown in Fig. 2. Duplicate standard curves were analyzed with each set of data collected. The relative error of the standards was less than 5% at all

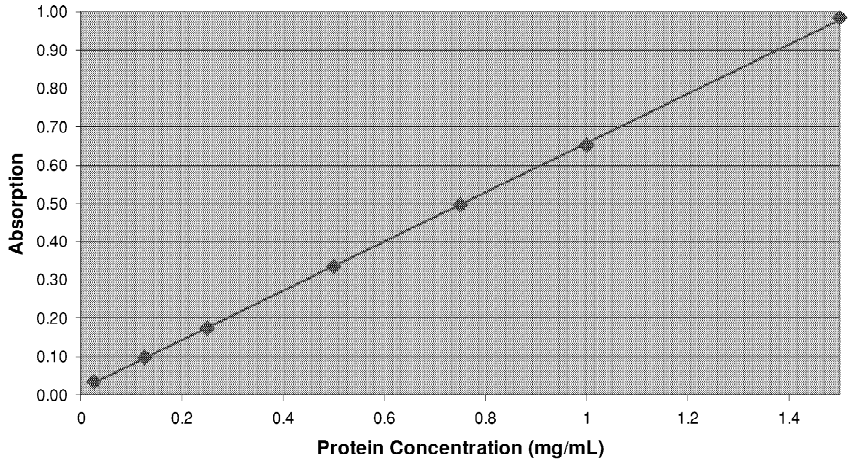

Fig. 2. Typical standard curve for bovine serum albumin protein standards (0.025–1.5 mg/ml) at 280 nm.

values. The relative standard deviation was less than 4%.

The protein precipitation results for the seven precipitants in dog, mouse, rat and human plasma are shown in Tables 1–4. Each protein precipitation efficiency value is an average of three replicates. A high protein precipitation efficiency results from a very low value of protein measured in the supernatant, and in some cases this was below the lowest standard of the standard curves analyzed with the samples (equivalent to >99.9% protein precipitated). Some points were rejected as outliers based on the Q-test for a 90% confidence interval; most of these were supernatants with very low protein concentrations and high protein precipitation efficiency.

With the exception of ammonium sulphate and aluminum chloride, all precipitants were on average at least 90% effective at a precipitant to plasma volume ratio of 2:1. Precipitant effectiveness increased with volume to a maximum reached at a ratio of approximately 2 or 2.5 to 1. Aluminum chloride was not used as a precipitant following the analysis of dog plasma due to its poor efficiency. Ammonium sulphate, despite being the most effective salt precipitant, is not commonly used in LC–MS bioanalytical preparations due to potential interface contamination. In this study, it was found to be an effective protein precipitant (~90%) at a minimum volume ratio of 2.5:1 (Tables 1–4).

Organic solvents are the most widely used protein precipitants utilized in drug analysis [1,14]. Acetonitrile was found to be the superior organic plasma protein precipitant, particularly at volume ratios <2:1 (precipitant:plasma). This study found ethanol and methanol to exhibit similar protein precipitation characteristics.

This study also showed that protein precipitation using strong acids and zinc sulphate (1:1 with 0.5 N NaOH) is effective at low volume ratios (Tables 1–4). This study indicates that effective protein precipitation (>90%) is observed at volume ratios of 0.5:1 for TCA.

Table 1
Comparison of protein precipitation efficiency for various protein precipitants in dog plasma

Precipitants		% Protein precipitation efficiency[a]						
		Ratio of precipitant to plasma						
		0.5:1	1:1	1.5:1	2:1	2.5:1	3:1	4:1
Acids	TCA	97.1	96.9	97.4	97.3	97.3	97.3	97.3
	%RSD	3.14	0.80	4.78	4.59	5.97	3.77	3.08
	m-Phosphoric acid	59.8	89.7	89.4	95.9	95.9	96.3	96.2
	%RSD	5.29	4.27	6.55	2.46	8.49	1.97	3.00
Metal ions	Zinc sulphate	82. 6	97.8	96.4	98.1	98.4	98.9	98.8
	%RSD ($n=3$)	17.40	17.27	12.32	19.05	9.62	–[b]	7.65
Organic	ACN	28.9	91.6	96.1	96.4	97.7	96.6	97.2
	%RSD ($n=3$)	8.36	5.17	2.50	4.31	2.46	0.64	4.01
	EtOH	6.4	69.2	86.3	89.7	94.4	93.7	93.8
	%RSD ($n=3$)	0.31	0.83	0.70	3.55	6.48	1.52	4.67
	MeOH	20.9	61.9	83.7	91.4	93.9	95.2	95.2
	%RSD ($n=3$)	7.59	4.27	4.75	2.24	4.17	2.84	3.58
Salts	Aluminum chloride	0	7.9	0.6	1.6	0.9	2	0
	%RSD ($n=3$)	2.79	11.10	2.07	7.44	3.55	5.83	0.03
	Ammonium sulphate	16.1	62.2	73.8	89.1	94.9	96.7	97.4
	%RSD ($n=3$)	2.11	5.30	10.75	2.93	3.18	3.46	–[b]

[a] % Protein precipitation efficiency = ([total plasma protein − protein remaining in supernatant]/total plasma protein) × 100.
[b] Two values obtained. One value rejected as an outlier based on Q-test for a 90% confidence interval.

Table 2
Comparison of protein precipitation efficiency for various protein precipitants in rat plasma

Precipitants		% Protein precipitation efficiency[a]						
		Ratio of precipitant to plasma						
		0.5:1	1:1	1.5:1	2:1	2.5:1	3:1	4:1
Acids	TCA	96.2	97.1	96.5	96.6	96.3	96.5	96.5
	%RSD ($n=3$)	9.31	5.38	7.16	4.38	4.88	11.25	11.18
	m-Phosphoric acid %RSD	Could not be determined due to cloudy supernatant						
Metal ions	Zinc sulphate	3.0	46.2	72.1	96.2	99.8	99.5	99.6
	%RSD ($n=3$)	7.65	3.39	11.26	17.11	–[b]	51.73	–[b]
Organic	ACN	54.4	94.8	96.7	97.1	97.1	97.6	98.5
	%RSD ($n=3$)	3.47	12.17	4.76	4.64	24.01	10.28	5.51
	EtOH	31.9	77.1	94.3	95.0	95.3	95.7	95.2
	%RSD ($n=3$)	7.01	4.66	2.63	15.23	7.13	7.14	14.30
	MeOH	9.4	65.0	93.6	96.4	96.9	97.6	>98
	%RSD ($n=3$)	0.73	2.75	1.92	4.68	11.29	3.77	–[c]
Salts	Ammonium sulphate	15.0	64.1	89.0	87.5	89.6	97.3	96.6
	%RSD ($n=3$)	3.62	3.71	7.94	55.54	0.08	8.09	4.27

[a] % Protein precipitation efficiency = ([total plasma protein − protein remaining in supernatant]/total plasma protein) × 100.
[b] Two values obtained. One value rejected as an outlier based on Q-test for a 90% confidence interval.
[c] Concentration of protein in supernatant below quantification limit.

Protein precipitation using metal-ion (zinc sulfate:0.5 N NaOH) precipitation is a very efficient process [17] and in this study, effective precipitation (>90%) was observed at volume ratios of 1:1 or greater for most species (Tables 1–4). Further, metal-ion precipitation yielded supernatants free of any particulate matter, an important factor for rugged assay performance.

When using either acidic or zinc sulfate:0.5 N NaOH precipitants, an evaluation of analyte stability is required due to potential degradation of analyte, degradation of acyl glucoronides at basic pH and analyte losses through coordination with zinc cations.

This study is in general agreement with data previously reported [5] using the Lowry method for assessment of protein precipitation effectiveness. Of the precipitants tested, acetonitrile, trichloroacetic acid and zinc sulphate provided the most efficient precipitation when compared against precipitants with similar mechanisms. Differences between this study and Blanchard's study may stem from the use of different protein quantification methods. The Lowry method is much more sensitive to protein, although less robust against interfering substances [2]. In the study reported here, where correlation for nucleic acid interference is measured, a consistently lower result for protein precipitated is reported using all precipitants chosen when compared against literature values [5]. In this study, on average 90% plasma protein precipitation is observed using volume ratios of 2:1 precipitant to human plasma, compared to the values >99% previously reported [5].

This study assessed inter-species variability for dog, rat, mouse and human plasma when performing in protein precipitation studies (Tables 1–4). Only minor differences in protein precipitation were seen in the three animal species chosen when using TCA as a precipitant. Consistently lower efficiency was observed across all volume ratios tested when precipitating human plasma with TCA.

The use of *m*-phosphoric acid as a precipitant presented another challenge with regards to species differences. The rat and mouse supernatants were

Table 3
Comparison of protein precipitation efficiency for various protein precipitants in mouse plasma

Precipitants		% Protein precipitation efficiency[a]						
		Ratio of precipitant to plasma						
		0.5:1	1:1	1.5:1	2:1	2.5:1	3:1	4:1
Acids	TCA	97.2	96.2	96.4	96.4	96.6	96.7	97.3
	%RSD ($n=3$)	7.91	20.60	3.62	7.53	5.51	6.16	11.33
	m-Phosphoric acid	Could not be determined due to cloudy supernatant						
	%RSD							
Metal ions	Zinc sulphate	20.9	97.8	98.4	97.7	98.4	>99	>99
	%RSD ($n=3$)	9.22	9.59	20.36	13.50	25.8	–[b]	–[b]
Organic	ACN	1.7	92.8	97.2	97.8	98.3	98.4	98.7
	%RSD ($n=3$)	2.54	2.17	5.01	2.39	4.74	5.19	0.27
	EtOH	31.2	58.0	78.4	92.3	95.4	96.4	97.8
	%RSD ($n=3$)	2.84	6.80	3.44	3.96	3.70	4.75	4.25
	MeOH	10.1	56.8	82.0	93.5	95.6	96.8	96.3
	%RSD ($n=3$)	9.55	8.29	5.93	2.68	6.84	4.91	4.32
Salts	Ammonium sulphate	10.8	38.0	77.0	84.7	92.6	97.0	94.7
	%RSD ($n=3$)	1.257	1.23	1.30	25.07	31.32	2.41	24.98

[a] % Protein precipitation efficiency = ([total plasma protein − protein remaining in supernatant]/total plasma protein) × 100.
[b] Concentration of protein in supernatant below quantification limit.

cloudy and particulate. These samples could not be accurately assayed for protein content using spectrophotometry without filtration, which may lead to drug losses through adsorption and thus increased error. These observations were not specific to a single lot of plasma and this problem was specific to the rodent species tested. As a result, it is recommended that the use of *m*-phosphoric acid in some animal species be limited. Given that TCA precipitated plasma proteins more effectively, did not yield any cloudy supernatants and exhibited better lot-to-lot reproducibility (Table 5), TCA should be the first choice when an acid precipitant is desired based on protein precipitation efficiency alone.

Inter-species variability in the protein precipitation effectiveness of ethanol, ammonium sulfate and zinc sulphate:0.5 N NaOH was observed at low volume ratios (less than 1.5:1), where protein was far from effectively precipitated (<90%). At volume ratios greater than or equal to 2:1, good inter-species correlation was observed with all precipitants.

Four different lots of human plasma were treated with protein precipitants to determine the extent of lot-to-lot variability in the protein precipitation efficiency at volume ratios of 2:1 (Table 5). The relative standard deviations for inter- or intra-lot variability using human plasma were less than 5% for the precipitants evaluated. The greatest variation was noted for *m*-phosphoric acid with a relative standard deviation of ~5%. Further, of the precipitants tested, zinc sulphate, acetonitrile and TCA precipitated the most protein and demonstrated good reproducibility (RSD<1%).

The second portion of this study concentrated on the use of a protein bound drug, compound A, as an example for ionization effect studies following protein precipitation with various standard reversed-phase LC conditions. This compound, containing a carboxylic acid and imine functionality, is extensively protein bound (>90%) and has low aqueous solubility (<1 mg/ml).

The set-up for measurement of ionization effect due to co-extracted plasma components remaining after protein precipitation is based on the protocol described by Miller-Stein et al. [19]. Perturbations in some of the mass chromatograms at 4.2 min are due

Table 4
Protein precipitation efficiency of protein precipitants in human plasma

Precipitants		% Protein precipitation efficiency[a]						
		Ratio of precipitant to plasma						
		0.5:1	1:1	1.5:1	2:1	2.5:1	3:1	4:1
Acids	TCA	91.4	91.8	91.5	91.0	91.2	91.3	91.4
	%RSD ($n=3$)	4.46	–[b]	3.46	0.20	2.18	5.98	3.96
	m-Phosphoric acid	89.4	90.5	90.3	90.2	90.7	90.5	90.0
	%RSD ($n=3$)	1.48	4.56	3.52	3.23	12.36	2.35	6.23
Metal ions	Zinc sulphate	89.2	96.8	96.8	99.0	99.0	99.0	>99.9
	%RSD ($n=3$)	14.73	7.16	14.58	1.70	8.04	3.42	–[c]
Organic	ACN	3.6	88.7	91.6	92.1	93.2	93.5	94.9
	%RSD ($n=3$)	3.62	2.50	3.63	3.13	5.29	5.91	1.82
	EtOH	0.1	78.2	87.2	88.1	89.8	91.8	92.0
	%RSD ($n=3$)	2.85	2.43	1.65	9.47	9.56	2.46	1.06
	MeOH	13.4	63.8	88.2	89.7	90.0	91.1	91.5
	%RSD ($n=3$)	0.95	3.09	3.54	3.50	2.84	5.09	2.46
Salts	Ammonium sulphate	24.8	50.1	64.0	84.2	90.4	90.4	89.0
	%RSD ($n=3$)	1.80	4.37	3.61	0.53	7.11	3.74	2.45

[a] % Protein precipitation efficiency = ([total plasma protein − protein remaining in supernatant]/total plasma protein) × 100.
[b] One value obtained. Samples discarded in error prior to assay.
[c] Concentration of protein in supernatant below quantification limit.

to a valve switch at the end of the autosampler run. This step was later removed upon modification of the autosampler run-time.

Initial observations using both zinc sulphate and ammonium sulphate were halted due to excessive source contamination and a precipitous drop in instrument sensitivity without eluent diversion. It was found that solvent diversion for the first 90 s circumvented this (data not shown). However, this would bias the data reported and thus, these precipitants were not studied further in terms of ionization effect. A key benefit for salt or metal-ion precipi-

Table 5
Comparison of protein precipitation efficiency of precipitants in different lots of human plasma

Precipitants (2:1 ratio)		% Protein precipitation efficiency[a]					Mean ($n=5$)	%RSD ($n=5$)
		Lot number						
		1[b]	1	2	3	4		
Acids	TCA	91.0	91.4	91.9	91.2	90.8	91.3	0.41
	m-Phosphoric acid	90.2	89.3	79.8	87.7	81.5	85.7	4.94
Metal ions	Zinc sulphate	99.0	98.5	98.5	99.0	99.1	98.8	0.27
Organic	ACN	92.1	93.1	93.9	93.5	93.4	93.2	0.65
	EtOH	88.1	88.1	89.1	88.4	89.2	88.6	0.54
	MeOH	89.7	86.9	88.2	89.2	89.3	88.7	1.14
Salts	Ammonium sulphate	84.2	84.8	85.8	86.1	87.1	85.6	1.17

[a] % Protein precipitation efficiency = ([total plasma protein − protein remaining in supernatant]/total plasma protein) × 100.
[b] Same matrix lot, assayed separately.

tation is the ability to precipitate plasma proteins without generating an elutropic supernatant, thereby facilitating more sensitive analysis using greater injection volumes for LC–MS/MS. Conservation of MS interface integrity requires a solvent divert to waste to reduce involatile salt build up. Further, appropriate chromatographic retentive capacity is also required. The long-term chromatographic effect of the basic nature of the metal-ion precipitated sample should be evaluated.

The ionization effects on compound A as a function of mobile phase composition and human plasma protein precipitant (2:1 precipitant:plasma ratio) using the mass spectrometer compatible precipitants were evaluated (Table 6). The mobile phases were varied to determine the effects of changing organic content, nature of organic solvent, ionic strength and pH on the extent of ionization effect when performing human plasma protein precipitation. The acquisition time was 10 min for all mobile phase/precipitant mixtures.

In our laboratory, bioanalytical LC–MS/MS methodologies are developed with ionization effects less than 20% to allow for variability in sample analysis. The data presented here indicate that a number of precipitant/mobile phase mixtures are unsuitable due to ionization effects in excess of 20%. In particular, organic precipitants with pure solvent mixtures exhibit significant ion suppression in excess of 68%. The use of acidic precipitants with pure mobile phases yielded ionization enhancement of less than −9.7%, thus overcoming the severe ionization effects observed when using organic precipitants. This may be due to enhancement of ionization of compound A in relation to endogenous substances.

Variation in mobile phase elution strength (elutropic composition and organic content) yielded similar results in terms of ionization effect for the different precipitants employed. As expected, the duration of ionization effect was reduced as a function of increased mobile phase elution strength. Further, the duration of ionization effect was reduced when using acidic precipitants with pure mobile phase solvents.

An example of the ionization effect observed for methanol, ethanol, acetonitrile, trichloroacetic acid and *m*-phosphoric acid protein precipitants in 50:50 methanol:water is shown in Fig. 3. The extent and duration of ionization suppression was considerable

Table 6
Measurement of ionization effect with various protein precipitant and mobile phase combinations in human plasma

Mobile phase	Acetonitrile		Methanol		Ethanol		Trichloroacetic acid		*m*-Phosphoric acid	
	Ionisation effect (%)	Duration (min)	Ionisation effect (%)	Duration (min)	Ionisation effect (%)	Duration (min)	Ionisation effect (%)	Duration (min)	Ionisation effect (%)	Duration (min)
50:50 Methanol:water	76.0	0.4–7.0	85.8	0.4–7.7	86.3	0.4–7.7	−3.0	0.5–1.0	0.3	0.5–1.0
50:50 Acetonitrile:water	68.2	0.5–6.4	92.0	0.5–2.6	85.9	0.5–2.6	−9.7	0.5–1.0	−8.1	2.0–5.0
70:30 Methanol:water	83.2	0.5–5.2	90.9	0.5–5.2	93.0	0.5–5.2	−2.0	0.5–1.5	−8.3	0.5–1.5
50:50 Methanol:0.1% formic acid	−2.9	0.5–2.0	−4.0	0.5–2.5	−2.1	0.5–2.7	13.6	0.5–2	1.0[a]	0.5–1.3
50:50 Methanol:10 m*M* ammonium formate	−194.6[b]	0.5–9.9	52.3	0.5–9.9	−20.2	0.5–9.9	14.7	0.5–9.9	−19.6	0.5–9.9

Analyte retention times of 8.0, 0.7, 0.4, 12.0 and 17.6 min using 50:50 methanol:water, 50:50 acetonitrile:water, 70:30 methanol:water, 50:50 methanol:0.1% formic acid and 50:50 methanol:10 m*M* ammonium formate mobile phases, respectively.

[a] Measured for 1.3 min only.

[b] Measured following interface cleaning between blank and human precipitated plasma samples.

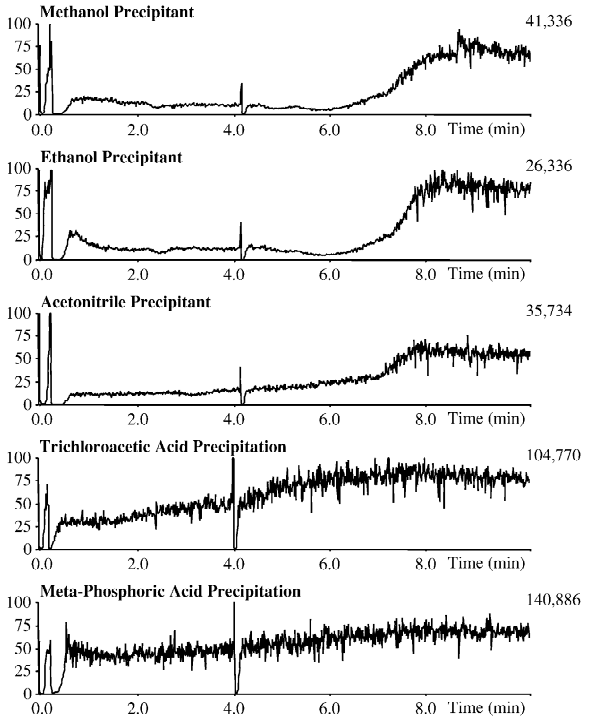

Fig. 3. Comparison of ionization effect using various precipitants in human plasma (2:1 vol. ratio) with a 50:50 methanol:water mobile phase.

when using organic precipitants. Of the organic precipitants evaluated, duration of suppression was similar. However, acetonitrile consistently exhibited the lowest ionization suppression when using pure mobile phase solvent mixtures, and thus is the organic precipitant of choice in terms of ionization effect. It should be noted that the increased elutropic strength of acetonitrile precipitated samples relative to methanol and ethanol should be evaluated, particularly when using large injection volumes and isocratic LC methodologies with typical bioanalytical LC columns (50×2 mm internal diameter).

Acid precipitants enhanced the intensity of the signal by 2–5-fold and did not show any significant ionization effect (>20%) from remaining plasma components. The total ion chromatograms for both acid precipitants exhibited ionization effects from 0.5 to 6 min, however, this was also observed within the blank samples and may be due to ionization effect produced by the precipitants. The use of a solvent divert would be advisable for both of these precipitants and an evaluation of the effect on chromatographic performance over time is essential when precipitating using low pH solvents (TCA precipitated supernatant pH 2.0 at a 1:1 ratio), particularly when using potentially labile stationary phase columns (e.g. cyanopropyl functionalities). Analogous to the usage of salt precipitation, a potential benefit of acid precipitation is the ability to increase the injection volume without compromising chromatographic efficiency and thus, improve assay sensitivity.

In general, inclusion of aqueous mobile phase additives reduced the extent of ionization effect. Ionization suppression observed using organic precipitants with pure mobile phases was overcome using acidic mobile phase components (50:50 methanol:0.1% formic acid). Improved ionization efficiency was observed when using 50:50 methanol:0.1% formic acid as a mobile phase, relative to 50:50 methanol water, which may account for the reduction of ionization suppression. Reductions in signal perturbation were observed when using an increased ionic strength mobile phase (50:50 methanol:10 m*M* ammonium formate). While an ionization effect was observed for each precipitant, the ion current was completely stable throughout the acquisition time of the experiment, hence, the duration of ionization effect values from 0.5 to 9.9 min (Table 6 and Fig. 4).

In bioanalytical LC–MS/MS, chromatographic properties are modified to ensure the retention time of the peak of interest falls outside of a range of significant ionization effect. Further, in our laboratory, an ionization effect greater than 20% is unacceptable. Thus, comparing the duration of ionization effect and also the retention time of the analyte under the mobile phases chosen (Table 6), it is clear that both 50:50 acetonitrile:water and 70:30 methanol:water are inappropriate choices for initial LC conditions due to the coincidence of retention time and ionization effect. The choice of 50:50 methanol:water or 50:50 methanol:0.1% formic acid as final mobile phase compositions enables resolution of analyte retention time from ionization effect, although further development is required to improve the assay cycle time. The data generated using 50:50 methanol:10 m*M* ammonium acetate is inconclusive as the analyte signal did not return to initial steady

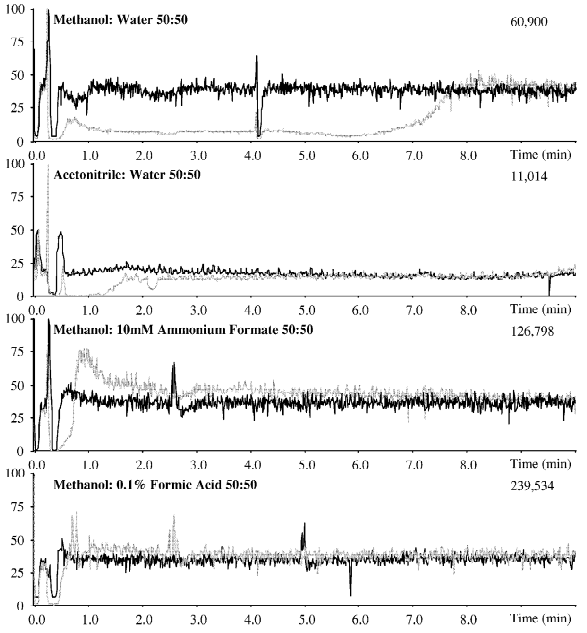

Fig. 4. Comparison of ionization effect with various mobile phase compositions following ethanol precipitation of human plasma (gray) and blank samples (black) (2:1 precipitant:sample ratio).

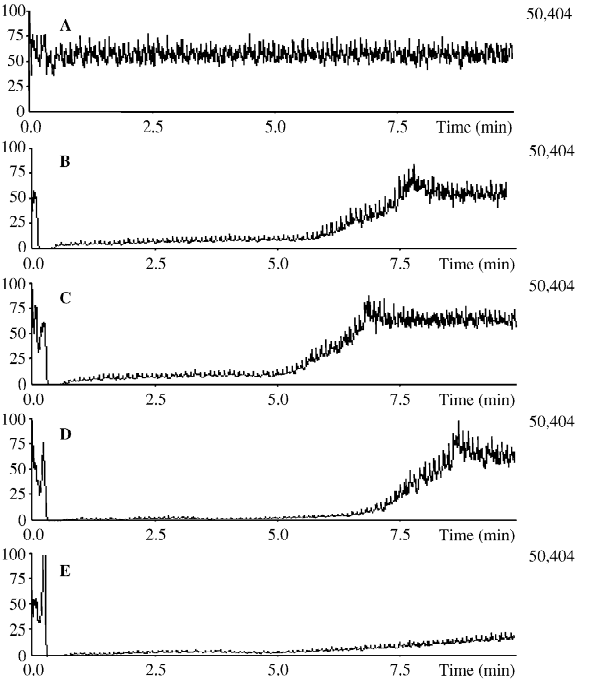

Fig. 5. Variation in ionization effect for acetonitrile precipitant added to water (A), human plasma (B), dog plasma (C), mouse plasma (D) and rat plasma (E) (2:1 vol. ratio) with a 50:50 methanol:water mobile phase.

state during the 10-min acquisition window (Table 6).

Variation in the extent of ionization effect across different plasma types was observed (Table 7). The ionization effect in 50:50 methanol:water with human, dog, mouse and rat plasma following acetonitrile precipitation is shown in Fig. 5. A difference was observed in the duration of ionization suppression for rat plasma relative to human, dog and mouse plasma samples. The analyte signal did not return to steady-state during the 10-min acquisition for rat plasma (Table 7). These results, in combination with the species variation found in *m*-phosphoric protein precipitation, suggested a difference in plasma components between rat and mouse versus dog and human.

Table 7
Variation of ionization effect with plasma species using acetonitrile precipitation (2:1 vol. in 50:50 methanol:water)

Plasma type	Ionization effect (%)	Duration of ionization effect (min)
Human	86.9	0.5–7.5
Dog	86.5	0.5–6.8
Mouse	92.6	0.5–8.6
Rat	92.9	0.5–9.9[a]

[a] Ionization suppression evident throughout the entire region of analysis. Steady state signal achieved after 12.8 min (data not shown).

Major concern occurs when the retention time of the drug of interest lies within a region of ionization suppression or enhancement, as drug response can be affected dramatically. The analysis of organic precipitated samples of compound A was found to be inappropriate in 50:50 acetonitrile:water and 70:30 methanol:water due to analyte elution within the region of ionization suppression. For proper quantification of a drug using protein precipitation followed by LC–MS/MS, the appropriate balance between the degree of ionization suppression or enhancement in the given mobile phase, effectiveness of the protein precipitant, analyte retention time and peak shape must be achieved. Further considerations will be required in developing an analytical method, such as, analyte stability under pH and solution conditions,

analyte organic and aqueous solubility, metal coordination to the analyte, and compatibility with the LC–MS/MS system.

Overall, with certain precipitants it is essential to utilize certain minimum volumes of precipitant, but beyond these minimums there are not extensive differences in the amount of protein precipitated. This may account for some of the considerable variation found within the literature on the precipitant volumes in use.

4. Conclusions

The most efficient protein precipitants for protein removal were found to be zinc sulphate, acetonitrile and trichloroacetic acid. These three precipitants consistently removed plasma protein effectively in all species and at all precipitant to plasma volume ratios of 2:1 and greater. At 2:1 volumes of precipitant to plasma, zinc sulphate removed 96% of plasma proteins, acetonitrile removed 92% of plasma proteins and trichloroacetic removed 91% of plasma proteins (averages of three replicates across four species). Further, these three precipitants exhibited excellent protein precipitation reproducibility (RSD$<$1% using five replicates) following precipitation of human plasma.

With the exceptions of *m*-phosphoric acid and aluminum chloride, the chosen protein precipitants can be applied universally to varying species and lots at volume ratios of 2.5:1 for protein removal in excess of 90%. Significant inter-species variability was observed when using *m*-phosphoric acid for precipitation of rat and mouse plasma (cloudy supernatant) compared to human and dog plasma samples. We did not find aluminum chloride to be an effective plasma protein precipitant when used as a 5% (w/v) solution. More concentrated solutions were not investigated.

This study suggests that the use of pure mobile phases with organic precipitants results in the greatest degree of ionization effect. Further, while the inclusion of ammonium formate buffer reduced the ionization effect using organic precipitants, the overall effect was still in excess of 20%. It should be noted that similar mobile phase conditions are often chosen in open-access LC–MS/MS environments where ionization effect is often unchecked.

This study clearly indicated that the use of acidic components as protein precipitants had a significant effect on ionization efficiency and also on ionization effect. Protein precipitation using acidic precipitants overcame any potential ionization effect in all mobile phase buffer systems analyzed. Further, this study also indicated that although ionization suppressing components were extracted using organic precipitants, the ionization effect was overcome using an acidic mobile phase consisting of methanol:0.1% formic acid. The reduced ionization effect may be a function of the improved ionization efficiency for the analyte relative to co-extracted plasma components.

The optimal bioanalytical methodologies for removal of plasma proteins and minimal ionization effect for the probe molecule in positive ion turboionspray LC–MS/MS involve either the use of TCA for precipitation with mobile phases consisting of pure organic solvents (methanol:water or acetonitrile:water) or precipitation with any of the mass spectrometer compatible precipitants with a methanol:aqueous 0.1% formic acid mobile phase.

These findings allow bioanalytical laboratories to choose protein precipitants based not only upon their effectiveness in protein removal, but also on their compatibility with the remainder of the assay.

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