RESEARCH ARTICLE

Quality Assurance in Drug Assaying and Pharmacokinetics-Blood Protein Evaluation in Calibration Curves

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ABSTRACT

In bioanalytical drug assays, plasma protein/albumin content can challenge the biomatrix and reduce drug recovery through the albumin-binding affinity (ABA) of drug molecules. Global quality assurance in sample preparation for analyte quantification during bioavailability assessments has evolved extensively, and the quality standards of the strictly regulated current global quality controlled drug manufacturing processes (cGQMP) now apply in pharmacokinetics (PK) studies. Previous analyses in large clinical trials had found that laboratory-prepared calibrator plasma/serum protein levels differed significantly from those of patients with occasional hyperproteinemia/hypoproteinemia and disease-related hyperalbuminemia/hypoalbuminemia. We, therefore, investigated improving assay accuracy by including adjustments for patient plasma/serum protein levels in protein evaluation calibrations curves (PROTEC). Using a combined PROTEC of two calibrators (with 1.6 ± 0.5 g/dL and 4.3g/dL albumin, respectively) to test rifampicin recovery from patients with hypoalbuminemia (1.6 ± 0.5 g/dL), we found that relative accuracy of drug recovery differed by minimum 0.1% for low ABA drugs and maximum >20% for moderate ABA drugs. Assay accuracy improved after accommodating for varying patient plasma/serum protein levels. We, therefore, propose using patient-calibrator PROTEC-PK in validation assay development/therapeutic drug monitoring to ensure that patient albumin levels are within acceptable validation accuracy ranges.

Keywords: Accuracy, Albumin, Calibration curves, Plasma Protein content, Quality Assurance.

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INTRODUCTION

Quality control parameters and compliance limits are well researched and documented in journals and pharmacopoeias. As such, her,e we describe the alignment of bioanalytical drug assessments in the laboratory with physiological absorption, distribution, metabolism and excretion (ADME) processes by including patient protein levels in constructing calibration curves. In a bioanalytical laboratory, efficient processes, without sacrificing accuracy and precision, effectively combining ADME for various drug molecules, as confirmed by validation procedures. Yet, there is still less than 100% drug recovery evident in calibration curves. Validation protocols are comprehensive quality assurance documents outlining strategic methods and referenced detailed standard operating procedures. In our laboratory, these protocols are typically the kinds of tests for which we hereby support the US Food and Drug Administration (FDA) in proposing feasible and practical solutions for improving assay accuracy. Consequently, as international quality standards have become increasingly demanding, in this study, we show how to increase confidence in bioanalysis by improving the accuracy of the margin of error. To do this, we propose a quality assurance perspective in bioanalytical methods where validation of protein evaluation in calibration curves for pharmacokinetics (PROTEC-PK) is in keeping with patient plasma or serum protein levels. Once plasma protein levels are known, they can be used to provide a more accurate assay result. If it is confirmed that a drug binds to plasma proteins, it can be inferred that the amount of plasma protein will affect both the quantity of free drug and the amount of drug recovered during the analytical process. Individuals with high plasma protein content relative to the calibrator protein content will then produce lower levels of drug recovery during laboratory procedures.

We adhere to the International Standards Organization (ISO) and the US Health and Human Services/FDA's Guidance for Industry: Bioanalytical Method Validation that includes concerns regarding the matrix effect and problems with recovery during liquid chromatographic-mass spectrometry (LCMS) bioanalytical work.[1,2] The South African National Accreditation System (SANAS), which offers laboratory accreditation services, requires similar validation of procedures and conformity to these guidelines. Cooperation amongst multidisciplinary, international

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research hubs can, therefore, ensure high levels of accuracy and precision in drug quantification and give insight into the limits of a drug's chemical stability and reactivity during in vitro and *in vivo* studies.

Biological matrices often contain a large variety of biomarkers such as endogenous substances, oxidatively processed metabolites, and products of the treatment of opportunistic diseases with a co-administered drug(s). Since patient samples cause variations in matrices and their associated effects, the samples challenge validated methods in bioanalytical procedures, putting the methods at risk.[3,4] This is seen in the manner in which the calibration curve is affected by the presence of strongly bound drug-albumin complexes. Whole blood (WB), as a biological matrix, plays a vital role in the quantification of exogenous drug activity. Blood infected with human immuno virus (HIV) and malarial parasites, for example, could have an effect on the quantity and condition of plasma proteins. Similarly, other atmospheric and airborne substances can enter the systemic circulation. This was noted during the study of caterers and residents of informal settlements – where firewood was used as an energy source – when systemic levels of chrome, copper, and arsenate (CCA) were identified.[5] Although that study used urine samples, the CCA was absorbed and quantified. Environmental factors like air quality thus affect the content of a physiological matrix, meaning metals in patient blood samples could likewise enhance matrix effects during the liquid chromatography-mass spectrometry (LCMS) analysis.

Plasma viscosity (PV) is a significant analytical biomarker of hemorheology studies. Hemorheology and plasma-tissue equilibrium may arguably be significant factors in the accuracy of drug assays and therapeutic drug monitoring; however, hemorheology and plasma-tissue equilibrium will not be covered within the scope of this research. For our study, we consider PV as being mostly dependent on the protein-water ratio and, in that, we have focused on the protein component. Whole blood plasma viscosity (WB-PV) can be assessed by water content as the protein content to water content ratio reflects the plasma viscosity. In healthy individuals, the body's homeostatic capability regulates its hydration level because hydration generally varies with environmental conditions

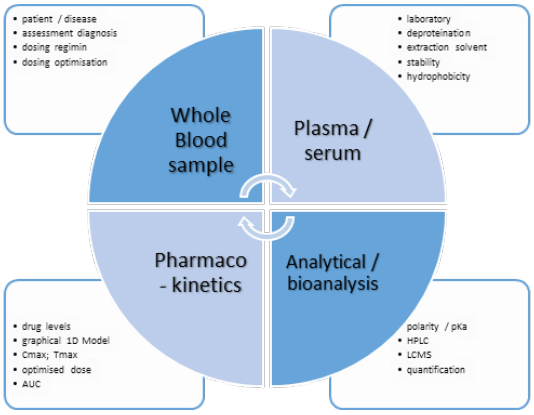

Figure 1: The process followed for dosing optimization in a PK study

and dietary (including fluids) intake. A patient's PV is also affected by disease and hydration levels; consequently, hyperproteinaemia and the efficacy of the homeostatic adjustments of plasma water content cannot be overlooked even though most patients will not have significant deviations from the normal blood water-protein ratio of approximately 12:1. However, changes in this ratio of up to 5:1 have been observed, and this confounds the error if the patient blood water-protein ratio is also affected by disease and oxidative metabolic products. The results of blood water level determinations can, therefore, be a red flag for further investigations to be conducted on patient protein levels and for the calibrator stock albumin levels to be assessed as well. We attribute the significance of WB content and multifunctional capability, including biomarking, to differences in patient PVs.

Biomarkers add to the complexity of the protein levels in WB. Pathologically, low-density lipoprotein and blood pressure are viewed as major determinants of the progression of coronary artery disease and, when elevated in WB samples, are useful biomarkers of coronary artery disease.[6] This increases the probability that a blood test sample could be used to identify positive cases of coronary artery disease, especially as acute coronary syndromes are expected to remain the leading cause of mortality and morbidity in the upcoming years.[7] Post-mortem studies have shown that acute coronary syndromes are mostly caused by thin-cap fibroatheroma lesions mostly cause acute coronary syndromes.[8,9] Similarly, plasma containing metabolites can play a role in the early detection of Parkinson's disease, which currently is often diagnosed by motor symptoms at latter stages of the disease. These metabolites are widely studied and also contribute to the complexity of the blood matrix.[10-22]

In our research, we aimed at exploring factors that influence the accuracy of drug assays and at identifying the most cost-effective and realistic drug bioanalytical methods during validation and therapeutic drug monitoring. Our main focus was the variations between the calibrator's plasma protein levels and the patient's protein levels in plasma or serum. In addition, because bioequivalence and bioavailability studies essentially require accuracy of the results as well, we merged scientific and clinical data into calibration curves using past achievements by researchers in this field. Calibration curves, representative calibrators, and the regression model have been defined previously in regulatory guidance documents, and by the experts.[23-33]

MATERIALS AND METHODS

The assay analysis was performed using LCMS. A typical analysis uses a positive mode; electron spray ionization interface-Shimadzu triple quadrupole mass spectrometer 8040 (MS) connected in tandem, using a two-channel (binary) pump, to a Shimadzu high-performance Liquid Chromatography (HPLC) system. The MS conditions were optimized for best sensitivity as follows: interface current of 0.1 µA, the nebulizing gas flow of 3 L/min, drying gas (N_2) flow of 15 L/min, desolvation line temperature of 250°C,

heat block temperature of 400ºC and CID gas (Argon) at 230 kPa.

The selection of the extraction solvent for deproteination depended on the stability of the molecule's polarity during bench work. Maintaining the molecule in the non-polar state was essential as we were using a C18 column and reverse-phase system. Following post-column ionization, the sample entered the MS, where the separated mass was transformed into an ionized atmosphere in which the ions were separated and fragmented in unique mass to charge (m/z) ratios.

The amount of free drug can be correlated to the amount of bound and unbound drug in WB depending on the availability of the specific plasma proteins. We investigated the patients' plasma protein levels because, when these vary significantly from that of the calibrators, a significant error occurs.

The drugs of interest were small molecules undergoing PK in the range < 2000 g/mol. The assay analyzed drug concentrations to trace levels from micrograms per milliliter (µg/ml) to sub-100 nanograms per milliliter (ng/ml). The results with the patient samples were extrapolated from a laboratory calibration curve constructed using spiked drug-free plasma calibrators. The drug-free plasma used in the laboratory was donated by the Tygerberg (Academic) Hospital, Bellville, Cape Town, South Africa.

A simple rapid water content determination, an application which – to our knowledge – has not been published before, was utilized to determine the water content of plasma (Newtonian fluid) by Karl Fischer (KF) auto-titration, using Hydranal (Merck, lot. HX85808105) as the titrating agent.

The reference standards rifampicin, isoniazid, ethambutol, pyrazinamide, and desacetyl-rifampicin were obtained from Sigma Aldrich. The plasma used was from blood donor patients and we obtained it from the Tygerberg Hospital. These were critically ill patients who had contracted opportunistic infectious diseases, such as tuberculosis (TB), and were possibly co-infected with HIV. All samples were prepared in a phosphate buffer system (PO_4^{3-}, NaCl, and KCl from Sigma Aldrich). All reagents used were of an analytical grade, and Millipore distilled water was used throughout the experiments.

In support of the External Quality Assessment Schemes (EQAS), an accredited proficiency test laboratory to monitor the data was incorporated.

RESULTS AND DISCUSSION

We assessed plasma protein water content and modeled it against the calibration curves to ascertain the accuracy of the assays. We were primarily focused on the FDA's concern about the < 100% recovery in the bioanalytical method validation and its effects on assay results when analyzing drug levels in plasma or serum. We assumed that plasma or serum protein content was closely correlated with that of the patient's PV. Where a patient's therapeutic drug levels fall within a 0.1–25 µg/ml range, these usually need to be determined by chromatographic procedures, including HPLC-MS. The content of the healthy plasma is approximately 92% (m/v) water and 8% (m/v) protein. However, in this research, we were interested in the occasional protein outliers where protein content varies significantly from 8% to 28% i.e., 92-72% (m/v) water, and where recovery is below 100% in both the calibrator and sample extraction procedures. This may occur due to patient conditions affecting the PV such as hypoproteinaemia, hyperproteinaemia, hypoalbuminemia and hyperalbuminemia. Albumin concentrations were noted as severe hypoalbuminemia (<2.5 g/dL), moderate hypoalbuminemia (2.5-3.5 g/dL), normal (3.5-4.5 g/dL), and hyperalbuminemia (>4.5 g/dL). Although there are solvents than can ensure total lysis of protein, the drug structure needs to be maintained. As such, the extraction solvent used in HPLC-MS needs to ensure that the drug remains chemically intact and, where necessary, that the drug's polarity is suitable for the chromatographic technique. Unfortunately, this condition may not always ensure optimal deproteination.

Pharmacokinetic (PK) Data Showing Differences in Plasma Drug Levels

We used an excerpt from a typical clinical trial to show variations in patient PK, where the body interacts with the drug (ADME). We gauged the drug's efficacy by relating the plasma drug levels to the patient's recovery status (condition). We also looked at the therapeutic drug monitoring of TB and MTB patients with hypoalbuminemia (albumin < 2.5g/dL). The plasma pKa status of a drug determines whether or not the weakly acidic or weakly basic drug ionizes. Further, protein and tissue drug-binding affinities change as the electronic state of the drug molecule changes. These conditions can, however, be controlled in the laboratory to optimize the stability and recovery of the drug during analysis.

In Figure 3a, total drug (esomeprazole) plasma levels were analysed as a comparison to the rifampicin PK study. We took a special interest in the differences in the maximum drug levels (C_{max}) reached in a similar time (T_{max}) range (less than 2 hours). We assumed that the three patients (P1-P3) had the same protein concentration in their plasma. Therefore a single calibration curve, with the same plasma protein content calibrators, was usable. The low level of drug absorption seen could have been related to the drug formulation: for example, an enteric coating is added to the tablet where weak bases with high pKa's are taken orally. The PK curves are typical for an oral dose, high absorption weak bases, such as the proton pump inhibitor omeprazole (esomeprazole), which is rapidly absorbed and binds to the receptor of the H^+K^+-ATPase proton pump. The response duration spans a longer period than is indicated by the vascular esomeprazole levels in Figure 2a. Its primary pK_a of about 4.0, facilitates esomeprazole accumulation in the parietal cell, and, being a benzimidazole with a second pK_a of about 1.0, it is a prodrug that is activated by acids to a sulfenic acid or sulfonamide and can bind covalently with one or more cysteines of the ATPase.[34-35] The drug and its metabolites are thought to bind with stable covalent bonds to sites on the proton pump, and the recovery of these receptors is done over time. The obvious indicators for variations in patient physiology are the shifts in the PK curves and the areas under the curves (AUC), shown in Figure 2a, and these are related to

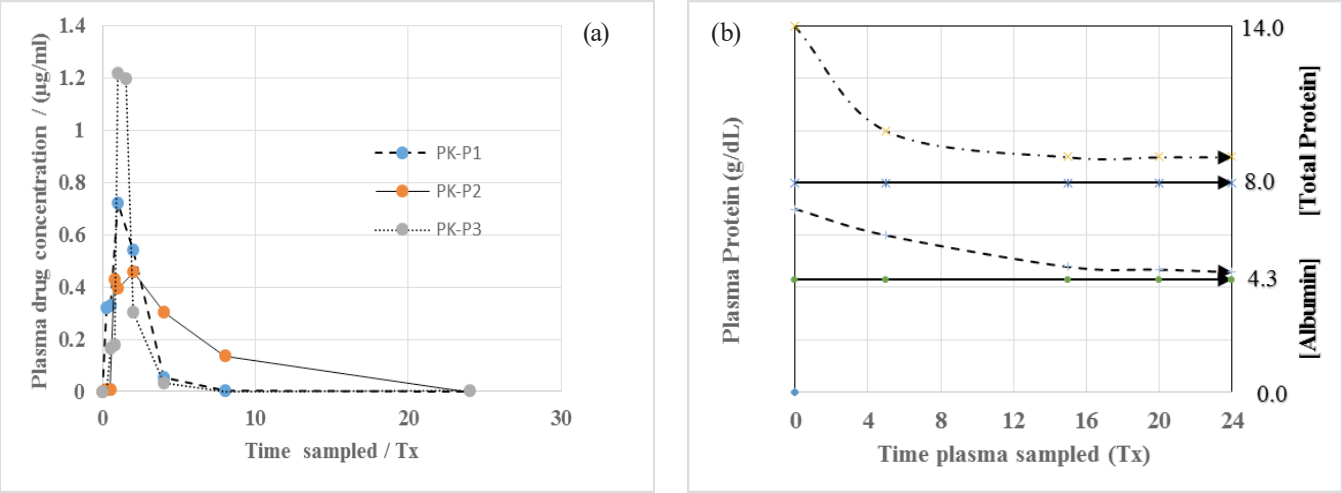

Figure 2: PROTEC-PK (a) Pharmacokinetic study of three patients with a non-polar drug (pKa 1.0 & 4.0); weak base). (b) PROTEC model for hyperalbuminemia and hyperproteinemia to normalization after an unspecified time, Tx.

variations in plasma protein content. We confirmed the *in vivo* drug-plasma interactions by assessing selected drug-protein binding energies and confirming variations in the physiology of individual patients. The improved accuracy of the drug assay was evident after accommodating the varying amounts of patient plasma protein.

In Figure 2b, the samples used were from pregnant patients, a low-risk cohort who were assumed to have normal to high plasma protein levels (hyperproteinaemia), and who were being treated for gastric acid reflux. During therapeutic drug monitoring or clinical trials, patient test sample variations in plasma protein levels are usually assumed to be low. The low-risk patient cohort were considered to be healthy as they were not admitted with symptoms of dehydration. As such, the amount of albumin used in the calibrators was assumed to be similar to that of the patients (4.3 ± 1.5 g/dL). A possible relative error could have been incurred by not knowing the plasma protein content of an outlying patient sample or by not taking it into account during the preparation of the calibrator. However, the relative error was presumed to be minimal and to not affect the accuracy of the assay relative to the drug recovery process. There would have been significant errors, however, if patients were experiencing hypoalbumenia or if the calibrators contained increased levels of albumin, exceeding the levels in the patients' plasma/serum.

The situation was different for the TB/HIV patients being administered rifampicin (Figure 3a). The patients generally admitted to the Tygerberg Hospital have low protein (albumin) contents around 1.6 ± 0.5 g/dL and below, whereas the normalized range of the National Health Laboratory Services (NHLS) is 3.5–5.2 g/dL. Hypoalbuminemia is usually associated with critically ill HIV- and TB-infected patients being treated with rifampicin. Rifampicin is soluble in methyl chloride with an octanol/water log K_{ow} = 4.24 (estimated) zwitterion, with pKa 1.7 due to the 4-hydroxy and pKa 7.9 due to the piperazine nitrogen.[36] This makes it only slightly soluble in water at pH <6, but, in the presence of hypoalbuminemia, a hydrophobicity change could alter the drug solubility levels in the blood. Therefore, with an increased water concentration in plasma in the hypoalbuminemic patients, the ionized

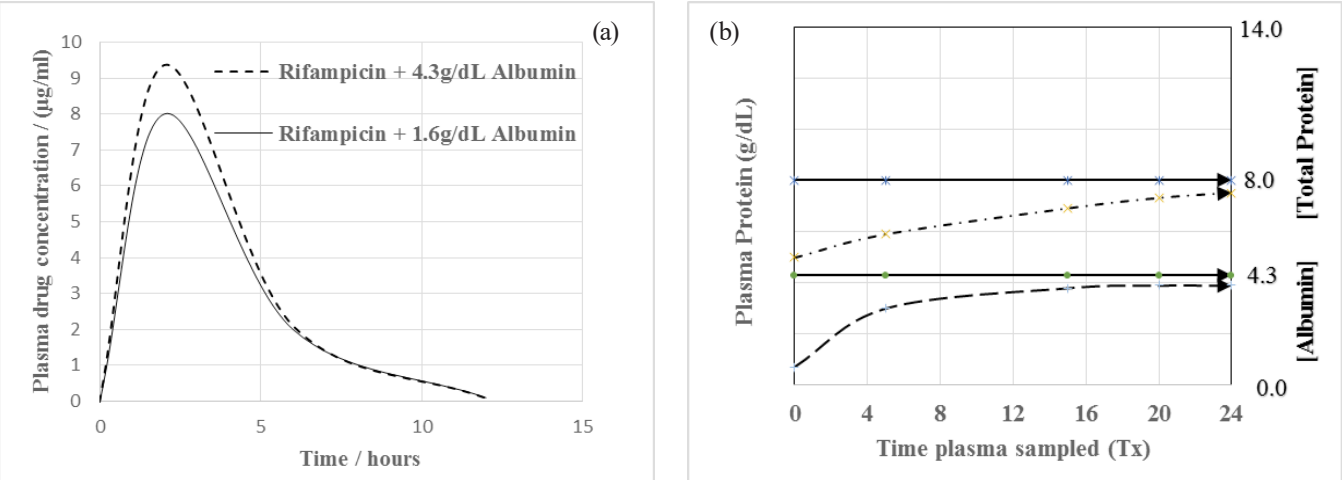

Figure 3: PROTEC-PK (a) Rifampicin (a weakly acidic non-polar drug) concentrations from two calibration curves containing 1.6 g/dL and 4.3 g/dL albumin where patient albumin is below normal albumin levels at 1.6 g/dL (b) PROTEC model for hypoalbuminemia and hypoproteinemia to normalization after an unspecified time, Tx.

rifampicin form may enter the renal elimination pathway giving the usually seen orange-tinted urine. The model in Figure 3b uses the protein evaluation calibrations curves (PROTEC) to show the stabilization of the protein and albumin levels during hyperalbuminemia and the PROTEC analysis for hypoalbuminemia. Analyses of free drug and bound drug in plasma can benefit from improved patient data collection at admission if blood protein levels are documented at that stage and subsequently sent through to the laboratory. Also, where possible, product information can be used to predict the drug-plasma interaction depending on whether or not tissue absorbance is likely or equilibrium of plasma proteins is achieved.

Figure 3a shows the corrected rifampicin levels using the PROTEC-PK (Figure 5a) for rifampicin as a weakly acidic non-polar drug. Two calibration curves were used in which the calibrators contained 1.6 ± 0.5 g/dL and 4.3 g/dL albumin (estimated), and the patient albumin level was below normal at 1.6 ± 0.5 g/dL (data supplied by the Tygerberg Hospital, NHLS). Figure 3b shows the patient total protein levels and the patient albumin levels (broken lines), which are in the 1.6 ± 0.5 g/dL region. The straight (solid lines) lines are indicative of normal total protein (8.0 ± 2.0 g/dL) and albumin (4.3 ± 2.0 g/dL) levels used in the calibrator preparation. Changes in PV from 12:1 to 5:1 (protein to aqueous ratio) in patient plasma and total plasma in calibrators, respectively, produce a more than 10% increase in the relative error. This would be significantly higher if the plasma protein (albumin) concentration increased in the calibrator stock without correlating it to patient albumin concentrations. The International Consortium for Harmonization of Clinical Laboratory Results has the role of reviewing priorities and maintaining a summary of measurement and harmonization activities of laboratories. Therefore, we used a test sample from an accredited laboratory to verify the proficiency of our results for rifampicin. Patient protein evaluation to determine the albumin binding affinity, especially where weakly acidic drugs are concerned, can assist in determining the competition for and spontaneity of drug binding to albumin, both of which affect the accuracy of patient-calibrator related drug recovery.

Figure 4a illustrates the rifampicin concentration ranges affected by protein (albumin) variation in the calibrators where < 100% recovery is noted. We investigated the patient sample with a low level (1.6 ± 0.5 g/dL) of plasma albumin that was incorrectly assessed for rifampicin levels using a calibration curve with normal, 4.3 ± 0.5 g/dL, albumin levels. The solvent calibration curve with zero albumin (0.00 g/dL) should correlate with a curve for 100% recovery, although this will vary for different drugs and laboratory equipment responses. At high calibrator drug concentrations, over 4µg/mL (Figure 5a), the differences in extrapolated results increase even as the amount of calibrator protein remains constant. If the total calibrator protein (plasma) content remains constant whilst the total diluted volume is maintained, the drug concentration is reduced by low-end calibrator formation. The deproteination solvent is also kept at a constant volume.

The model illustrated in Figure 4a shows the three possible drug concentrations for zero albumin, normal albumin – 4.3 g/dL (calibration curve), and patient protein (albumin) levels – 1.6 g/dL (calibration curve). The accuracy of the assay is in the differences between the drug concentrations obtained with the various albumin levels in the calibration curves. It can be seen that the accuracy is directly proportional to differences in the patient and calibrator protein levels in the calibration curves. The calibrator stock plasma protein distribution r^2 was 0.9999; however, the ratio of albumin to total protein was significant.

The gradient, m, for the curve (y = mx+c), was found to be directly affected by the drug-protein binding affinity and albumin levels. The detector response (y) was used in the extrapolation process. In Figure 4b changes in accuracy in drug concentration ranges A(0-2.49 µg/ml), B_1-B_4(2.49-12.5 µg/mL), C_1-C_2(12.5-17.5 µg/mL), D_1D_3(17.5-25 µg/ml) were

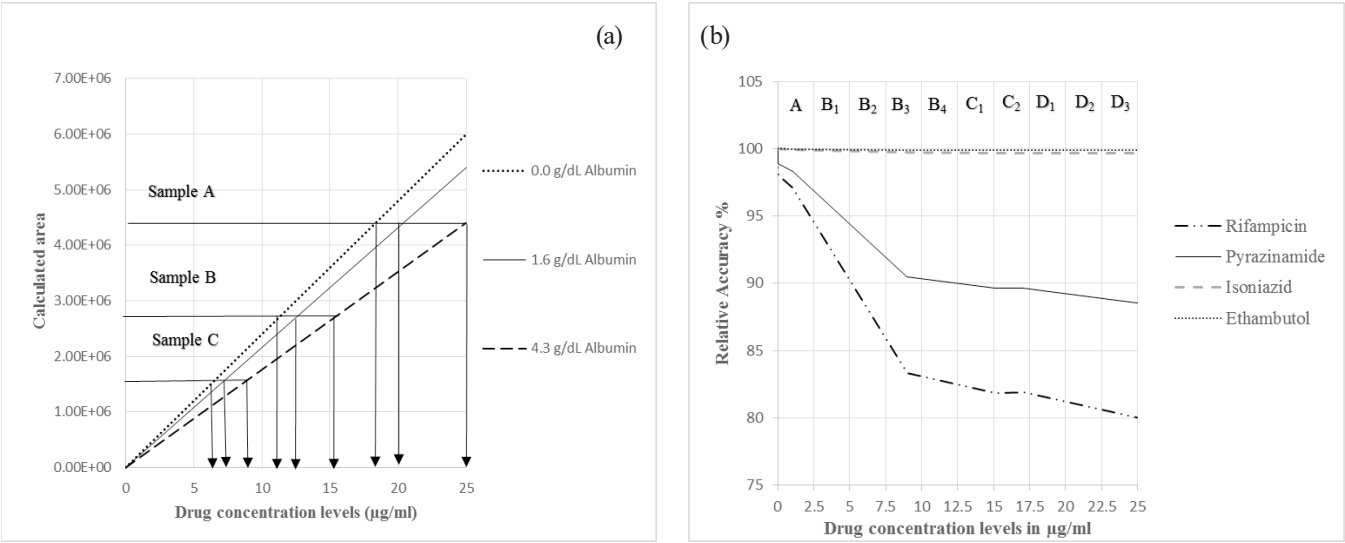

Figure 4: (a) Calibration curves with varied protein content and varying patient protein levels in plasma/serum (b) Modelled accuracy fluctuations for rifampicin-albumin MA binding affinity and the estimated isoniazid LA -albumin, ethambutol LA-albumin and pyrazinamide MA -albumin relative accuracy of binding affinities; (MA - moderate affinity and LA - low-affinity drugs).

noted. In Figure 4b, in region A, the accuracy is lowest as the low levels of the albumin-bound drug do not affect drug recovery by much. A gradual increase in relative error is visible after 2 µg/mL (regions B to D) with significant decreases in accuracy and increases in relative error noted from D_1 to D_3. In Figure 4a, low gradients (m) due to high albumin levels, cause an exaggerated increase in the extrapolated concentration (x), especially in the regions of higher concentration. Here we confirm variations in drug-protein binding and distribution by comparing conditions of normal albumin concentrations with hypoalbuminemia.

Figure 4b shows the relative accuracies (%) of the drug binding affinities of four drugs for protein (albumin). The model is based upon rifampicin's moderate affinity (MA) with the highest relative binding affinity for albumin. In the model, we estimated the accuracy of the drugs, considering the binding affinities relative to rifampicin. In supplementary information, we have already confirmed the binding affinities for albumin and the spontaneity of the binding for the four drugs, rifampicin, pyrazinamide, ethambutol and isoniazid. .

In Figure 4b, the drug properties are portrayed as relative to rifampicin's experimental binding energy data. As confirmed in previous research reported in the supplementary information, the binding energies were as follows: rifampicin 5.379 X 10^2 M^{-1} (moderate affinity), isoniazid 9.285 M^{-1} (low affinity), 25-desacetyl rifampicin 3.156 M^{-1} (low affinity), ethambutol 3.443 M^{-1} (low affinity) and pyrazinamide 3.076 X 10^2 M^{-1} (moderate affinity). The Gibbs free energies for the four drugs were below zero indicating spontaneous binding reactions. Rifampicin, a non-polar weak acid, has the highest binding affinity indicating that it will form more stable complexes with albumin as opposed to the soluble isoniazid, which being polar and in an ionized form, can be easily excreted in the urine resulting in low levels of detection. This affects the bioavailability and accuracy of assay levels for patients experiencing hyper- and hypoalbuminemia; rifampicin also influences competition and induction processes of the CYP3A4/5 enzymes. Dose optimization is further complicated where the drugs with low binding affinities have better recoveries and lower detector responses due to both low bioavailability and their being ionized as well. Isoniazid has low binding affinity and high recovery.[32] However the practice of crushing tablets and adding it to an aqueous medium could cause ionization and poor absorption leading to low plasma levels, which can also be misleading; thus, dissolution profiling is essential when altering regulatory approved formulation. For rifampicin, however, researchers have shown in their validation method that protein saturation probably occurs at increased rifampicin concentrations. Notably, unbound drug concentrations increase from 9% to 22% while protein binding decreases from 91% to 78% when the total drug concentrations rise from 1.7 to 64 µg/L.[37] This could explain the steady decrease in the gradient (slope) at a maximum of 25µg/ml when the albumin levels are increased. These complications are apparent in multidrug administration and where larger numbers of patients, more than 400, are involved in clinical trials. In these circumstances, the relative accuracy in bioequivalence and bioavailability studies, with varying patient protein levels, maybe more critical for drugs with a narrow therapeutic index. A detailed review of the diseases that contribute to the blood protein levels will be covered in a follow-up to this paper.

Drugs with low binding affinities for patient albumins, such as isoniazid, may become ionized and show high excretory levels if given with other drugs that have more competitive binding affinities for albumin. Compliance with FDA and international regulatory guidelines require reproducibility of a calibration curve that closely resembles calibrator and patient drug-protein interactions. PV (protein to aqueous ratio) changes from 12:1 to 5:1 in patient plasma and total plasma in calibrators caused the relative error to be increased by more than 10%. Additional increases in the difference between patient and calibrator albumin (protein) levels could produce a further 20% increase in relative error. Patients with hypoalbuminemia (or hypoproteinemia in cases of related low total protein levels) show the lowest accuracy in assay test results and have the highest risk of mortality. We, therefore, propose the use of the patient-calibrator PROTEC-PK in validation assay development and therapeutic drug monitoring to ensure that patient albumin levels are within acceptable validation accuracy ranges. Further validation of the binding energy determination is needed as blood plasma constituents other than proteins can affect the molecular docking and fluorescence quenching processes.

In conclusion, we support the need for harmonization, standardization, and evidential traceability of the next generation of clinical measurements as an essential mechanism in quality assurance. Our results also support the use of a representative calibrator matrix and albumin levels concerning the patient's albumin levels. The amount of drug-bound to albumin and the subsequent recovery levels affect the gradient (slope) of the calibration curve. Although we focused on rifampicin in the reported experiments, we can apply these protein assessments to other drugs, to minimize errors in optimizing accuracy in clinical trials, bioequivalence and bioavailability studies. This is significant and of concern where increases in drugs with narrow therapeutic index risk causing side effects and exceeding the C_{max}. Dose optimization needs accurate assays as low drug concentrations over extended periods may also cause drug resistance. Future validation should include other blood plasma constituent level determination to ensure fatty acids and other components are not offering binding sites for certain drugs as well.

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protein and albumin levels. Excerpts of the supplementary data will be submitted for publication at a later stage.

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