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Development of Protein-Specific Analytical Methodologies to Evaluate Compatibility of Recombinant Human (rh)IGF-1/ rhIGFBP-3 with Intravenous Medications Co-Administered to Neonates

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Development of Protein-Specific Analytical Methodologies to Evaluate Compatibility of Recombinant Human (rh)IGF-1/rhIGFBP-3 with Intravenous Medications Co-Administered to Neonates

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ABSTRACT

The protein complex of recombinant human insulin-like growth factor-1 and insulin-like growth factor binding protein-3 (rhIGF-1/rhIGFBP-3; mecasermin rinfabate), is an investigational product for the prevention of complications of prematurity. Delivery of rhIGF-1/rhIGFBP-3 is by continuous central line intravenous infusion in preterm infants until endogenous IGF-1 production begins. Protein-specific analytical methodologies were developed to evaluate the compatibility of rhIGF-1/rhIGFBP-3 at low protein concentrations (~2.5-10 μg/mL) expected when co-administered with other required medications in the NICU. Highly sensitive detection of the biologic potential degradants (fragments) and/or molecular modifications (oxidized species, aggregates) required the use of reversed-phase high-performance liquid chromatography and size-exclusion ultra-performance liquid chromatography coupled with mass spectrometric detection. We report on the quantification of rhIGF-1/rhIGFBP-3, its components and degradants, to a limit of quantitation of 3.1 μg/mL upon mixing with 24 commonly administered neonatal medications. Methods developed for the rhIGF-1/rhIGFBP-3 admixtures, optimized in studies with furosemide, caffeine citrate and ampicillin, demonstrated good reproducibility, linearity, and limit of detection/quantitation. Using these methods, no increase in degradation of rhIGF-1/rhIGFBP-3 components and no increase in oxidation or aggregation level was observed with caffeine citrate, while admixtures of rhIGF-1/rhIGFBP-3 with ampicillin yielded lower mass recovery of rhIGF-1/rhIGFBP-3 components, which likely resulted from adduct formation. Furosemide was found to be physically incompatible with rhIGF-1/rhIGFBP-3. Our findings support the use of these methodologies for detection of protein modifications under various clinical administration conditions, and additionally supplement physical compatibility data studies of ultra-low concentrations of rhIGF-1/rhIGFBP-3 post co-administration to preterm infants with other medications (manuscript in-preparation).

Introduction

Low IGF-1 levels in preterm infants, due to interruption of maternal-placental supply, may be associated with poor weight gain, impaired brain development, bronchopulmonary dysplasia, retinopathy of prematurity, and other morbidities.[1,2] rhIGF-1/rhIGFBP-3 (mecasermin rinfabate), an investigational product, is a recombinant human (rh) version of the naturally occurring protein complex of insulin-like growth factor-1 (IGF-1) and its binding protein,

Abbreviations: ACN, acetonitrile; HMW, high molecular weight; IGF-1, insulin-like growth factor-1; IGFBP-3, insulin-like growth factor binding protein-3; IPA, isopropanol; LOD, linearity of detection; LOQ, linearity of quantitation; MS, mass spectrometry; rh, recombinant human; RP-HPLC, reversed-phase high-performance liquid chromatography; RSD, relative standard deviation; SEC-UPLC, size-exclusion ultra-performance liquid chromatography; UV, ultraviolet; XIC, extracted ion chromatogram.

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insulin-like growth factor binding protein-3 (IGFBP-3), and is being studied for the prevention of complications of prematurity.[1,3,4] The complex is delivered by continuous central line intravenous (IV) infusion in preterm infants, usually for weeks, until endogenous IGF-1 production begins.

Due to limited IV central line access for neonates, concomitant administration of rhIGF-1/rhIGFBP-3 with other medications via a terminal injection site is likely. A biologic that requires central line infusion over weeks must be compatable with other required medications, which, depending on their formulation, may have significantly different physical and chemical properties. Furosemide (a loop diuretic prescribed for fluid overload), caffeine citrate (commonly prescribed for apnea of prematurity), and ampicillin (commonly prescribed to treat bacterial infections) are examples of a few of the commonly co-administered drugs with diverse pH and chemical reactivities that could be co-administered with rhIGF-1/rhIGFBP-3.[5-7] The consequences of co-infusing incompatible drugs may include precipitation, complex disassociation, change in color or pH formation of particulates, or gas evolution,[6,8,9] which could impact patient safety, particularly in preterm infants.[6] However, there are limited data on the chemical and physical compatibility of IV biologic drugs co-administered to preterm infants, and much of the current knowledge has been focused on physical compatibility.[5-8]

Analytical methodologies including reversed-phase high-performance liquid chromatography (RP-HPLC) and size-exclusion ultra-performance liquid chromatography (SEC-UPLC) coupled with mass spectrometric (MS) detection, have been or are being developed for monitoring the quality of rhIGF-1/rhIGFBP-3 after mixing with commonly used IV medications. A key consideration in assessing the compatibility of rhIGF-1/rhIGFBP-3 when co-administered with other medications is its extremely low protein concentration (~2.5–10 μg/mL),[1] which makes it difficult to detect potential degradants and/or molecular modifications; as such, detection requires the use of high-sensitivity techniques. Ultraviolet (UV) detection is commonly used with liquid chromatography (LC) separation to quantify individual species and degradants; however, the UV sensitivity was found to be insufficient to quantitate low-abundance degradants of the biologic drug at these ultra-low concentrations to truly represent the in-use conditions. A modified mass spectrometry-based version of each method was used in this study as it was highly sensitive and capable of quantifying individual species at the clinically relevant ultra-low concentrations at which rhIGF-1/rhIGFBP-3 is administered. The methods presented here are utilized to augment routine physical compatibility assessments, further inform the observed (or lack thereof) physical changes in terms of impact to the rhIGF-1/rhIGFBP-3 product, assess potential chemical incompatibilities, and evaluate any potential degradation route(s) for rhIGF-1/rhIGFBP-3 post mixing. Lastly, these data support an ever-evolving risk-based approach (manuscript in preparation) by potentially establishing distinct general governing principals for incompatibility.

Herein, we report on the development of two LC-UV-MS methods (RP and SEC) to detect and quantify components of the rhIGF-1/rhIGFBP-3 drug product and their degradants (oxidized species, fragments, by RP, and high molecular weight (HMW) species), by SEC, at ultra-low concentrations. These methods were then utilized to assess data for rhIGF-1/rhIGFBP-3 quality after mixing with furosemide, caffeine citrate, ampicillin, and other IV medications across therapeutic classes commonly used to treat complications of prematurity (**Fig. 1**).

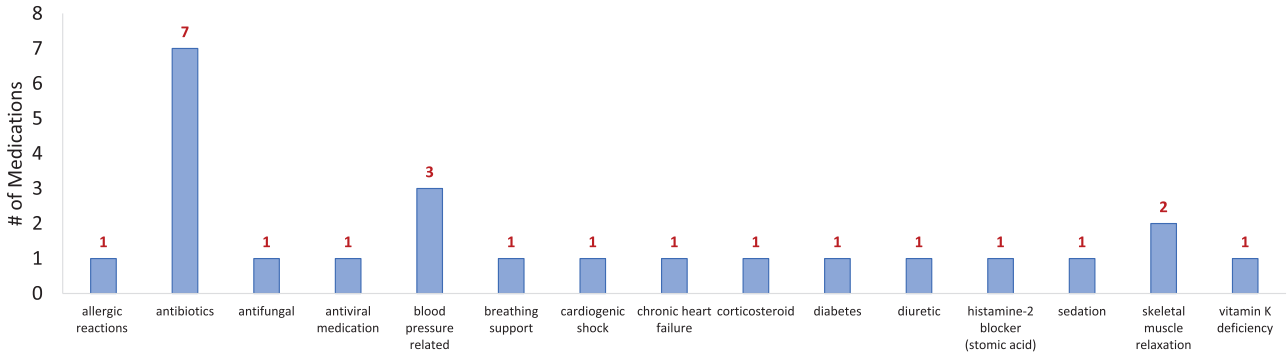
Methods and Materials

Sample Preparation, Processing, and Analysis

All samples and associated controls were prepared at room temperature. Standards and controls were pH matched to each medication, when needed. During sample preparation, small amounts of a surfactant were needed to reduce the non-specific adsorption of the product during handling and sample preparation.

rhIGF-1/rhIGFBP-3 Standard and Control Solutions

A 50 μg/mL solution of rhIGF-1/rhIGFBP-3 (in duplicate) in an acetate-based formulation at pH 5.5 with residual amounts of a commonly-used surfactant was used as the study and assay control. To determine limit of detection (LOD) and limit of quantitation (LOQ) of intact rhIGF-1/rhIGFBP-3, the 50 μg/mL solution was serially diluted with the formulation buffer to generate 25.0, 12.5, 6.3, and 3.1 μg/mL rhIGF-1/rhIGFBP-3 concentrations. The calibration curves were also constructed in the admixture and control samples for furosemide, caffeine citrate, and ampiciilin to establish the linearity. Upon



Category	Medication Name	Category	Medication Name	Category	Medication Name
Allergic reactions	Epinephrine	antifungal	Fluconazole	corticosteroid	Hydrocortisone sodium succinate
Antibiotics	Ampicillin	antiviral medication	Acyclovir	diabetes	Novolin R
	Vancomycin Hydrochloride	blood pressure related	Dopamine Hydrochloride	diuretic	Furosemide
	Penicillin G Sodium		Norepinephrine	histamine-2-blocker	Ranitidine
	Gentamicin		Duraclon (clonidine hydrochloride)	sedation	Precedex
	Cefotaxime	breathing support	Caffeine Citrate	skeletal muscle relaxation	Rocuronium Bromide
	Ceftazidime	cardiogenic shock	Dobutamine		Anectine
	Daptomycin	chronic heart failure	Soldactone (potassium canrenoate)	vitamin K deficiency	Kaytwo

Figure 1. Clinical medication categories for the 24 admixtures.

establishing the linearity among many studies, no further calibration curves were constructed in this manner.

rhIGF-1/rhIGFBP-3-drug Admixtures

For each of the rhIGF-1/rhIGFBP-3-drug admixtures, admixture solutions and controls were prepared to achieve specific clinically relevant concentrations (**Table S1**). Each medication was prepared per its packge insert, and if needed, diluted in its prescribed matrix (**Table S1**). The testing set for each medication at each concentration consisted of the rhIGF-1/rhIGFBP-3-drug admixture sample and a series of 2-6 controls, as needed (see **Fig. 2**). In cases of significant pH change post mixing compared with the release specification of the product (i.e. >0.3 pH units), pH matching of relevant controls was achieved either by dilution of rhIGF-1/rhIGFBP-3-drug with the matrix of the small-molecule drug or by adjusting the pH with a buffer system. The pH matching was conducted to generate a control to provide the impact of pH to rhIGF-1/rhIGFBP-3 in the absence of each small molecule. After mixing and pH adjustment, samples were equilibrated at controlled room temperature for at least 30 minutes prior to analysis to capture an estimate of the clinical mixing exposure time. After equilibration, admixture samples were divided into two aliquots: ~100 μL for LC-UV-MS analysis, and the remaining volume for pH measurement using a calibrated pH meter (Orion VersaStar Pro, Thermo Scientific).

rhIGF-1/rhIGFBP-3-furosemide Admixture for Method Development. rhIGF-1/rhIGFBP-3-furosemide admixtures containing 25.0, 12.5, 6.3, and 3.1 μg/mL of rhIGF-1/rhIGFBP-3 and 5.0, 7.5, 8.8, and 9.4 mg/mL of furosemide, respectively, were prepared in triplicate to generate appropriate calibration curves for the studies. No pH adjustments were needed for these admixtures.

rhIGF-1/rhIGFBP-3-drug Admixtures for Assessment of Method Performance. Across several medication categories, already tested for physical compatibility, eight neonatal drugs were first selected for admixing with rhIGF-1/rhIGFBP-3 and analyzed to assess the performance of the developed method with respect to accuracy, mass recovery, LOD, and LOQ. The rhIGF-1/rhIGFBP-3 admixtures included caffeine citrate, ampicillin monosodium salt, vancomycin hydrochloride, penicillin G potassium, gentamicin, hydrocortisone sodium succinate, cefotaxime sodium, and dopamine hydrochloride. Admixtures and corresponding controls each were analyzed in triplicate, and an additional sample set with varying rhIGF-1/rhIGFBP-3 concentrations also was prepared in triplicate for linearity testing (e.g. 12.5, 6.3, and 3.1 μg/mL), with the concentration range covering the to-be-tested rhIGF-1/rhIGFBP-3 concentrations in the admixtures.

When the pH-matched controls were needed for several rhIGF-1/rhIGFBP-3-admixtures, the pH adjustments were achieved using buffers to avoid direct titration of the rhIGF-1/rhIGFBP-3 solution with an acid or base. Sodium citrate buffers with varying pH values (4.0 –6.0) were used for admixture pH adjustment, including caffeine citrate (pH 4.7), vancomycin (pH 4.2), gentamicin (pH 5.2), hydrocortisone (pH 5.9), cefotaxime (pH 5.4), and dopamine (pH 5.2) admixtures, while glycine-NaOH buffer was used for ampicillin admixtures (pH 9.0). pH adjustment was not needed for the penicillin G admixture studies.

High-throughput Analysis of rhIGF-1/rhIGFBP-3 Admixtures with Additional Neonatal IV Medications. Sixteen small-molecule medications (drugs), already tested for physical compatibility, were selected across different medication classes for analysis using the protein-specific assays (dopamine, dobutamine, epinephrine, norepinephrine, fluconazole sodium chloride, dexmedetomidine (Precedex), ceftazidime pentahydrate, insulin (Novolin R), clonidine (Duraclon), potassium canrenoate (Soldactone), menatetrenone (KayTwo), acyclovir sodium, rocuronium, succinylcholine chloride (Anectine), ranitidine and daptomycin). rhIGF-1/rhIGFBP-3 admixtures with each medication, and associated controls, were prepared in duplicate (**Table S1**), and held at room temperature for 30 min prior to sample preparation for RP-HPLC-UV-MS analysis to allow for potential reactions to proceed.

Admixture Preparation for Analysis

Post-mixing and hold time, the drugs were removed from the biologic/drug admixture by resin–based desalting devices prior to analysis. A Zeba column (Thermo Zeba Spin 7K MWCO 0.5 mL desalting column, PI89882) was used for the RP-HPLC-UV-MS analyses while a Bio-Spin Column (Bio–Rad Bio–Spin P–6 Gel Column Tris Buffer, 7326228) was used for the SEC-UV-MS analyses as the former did not produce artifacts in the RP assay, while the latter did not produce

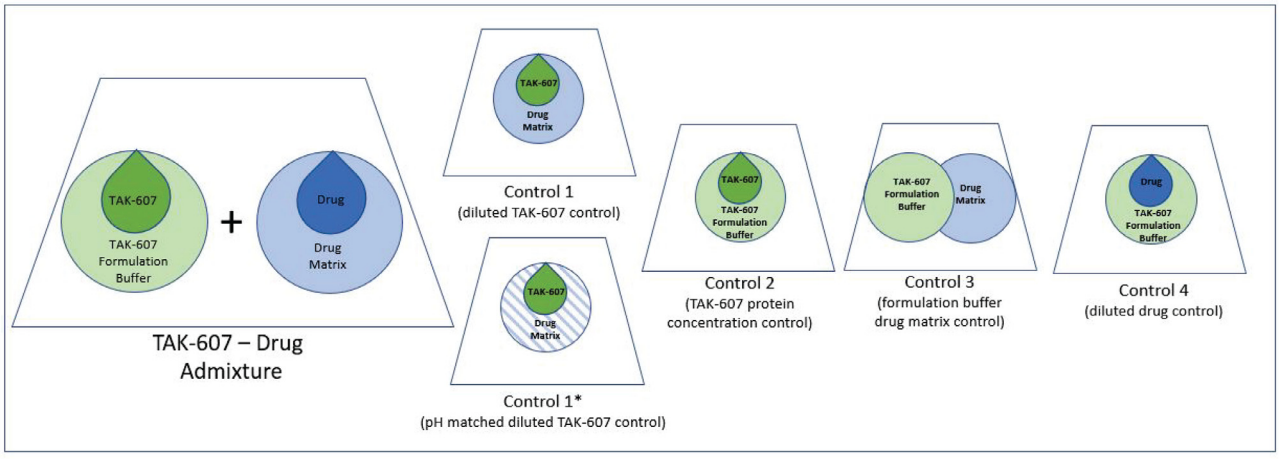


Figure 2. Schema for admixture and potential controls for the RP-HPLC-UV-MS studies. For the physical compatibility studies, the rhIGF-1/rhIGFBP-3-drug admixture at each concentration was prepared along with a series of controls, as needed; rhIGF-1/rhIGFBP-3 drug product controls were prepared by dilution of the drug product with the matrix of the drug (Control 1), and, in case of significant pH change post mixing (≥0.3 pH unit), one or more pH-adjusted rhIGF-1/rhIGFBP-3 drug product controls were generated by adjusting the pH with one or more relevant buffer systems of the drug matrix (Control 1*). The rhIGF-1/rhIGFBP-3 drug product was diluted with the rhIGF-1/rhIGFBP-3 formulation buffer to achieve a protein-concentration control (Control 2). A rhIGF-1/rhIGFBP-3 formulation buffer – drug matrix control was prepared (Control 3). Finally, one or more drug and pH matched rhIGF-1/rhIGFBP-3 formulation buffer mixture controls were prepared if needed (Control 4, Control 4*). RP-HPLC-UV-MS, reversed-phase high-performance liquid chromatography ultraviolet with mass spectrometric detection.

artificially higher percentages of HMW species in the aggregation analyses by SEC. The desalting columns differ in their resins: Zeba (polyacrylamide gel matrix) and Bio-Spin (proprietary mix), which may explain the absence of artifacts for their associated LC methods.

The Zeba and Bio-Spin columns were conditioned and equilibrated in a similar manner. Once the packing buffer was removed, the columns were conditioned by exchange buffer into a mixture of 15% organic mixture solution [75% acetonitrile (ACN), 20% isopropanol (IPA), and 5% LC-MS grade water] and 85% rhIGF-1/rhIGFBP-3 formulation buffer for the Zeba columns or 100% rhIGF-1/rhIGFBP-3 formulation buffer for the Bio-Spin columns by addition of 300 μL to the top of the resin bed, and centrifugation at 1500× *g* for 1 min for the Zeba columns and 1000× *g* for 2 min for the Bio-Spin columns. This was repeated three times to equilibrate the columns in their respective buffers. Then 300 μL of the appropriate buffer was added on top of the resin bed to avoid drying of the bed during the preparation of samples. Once the samples were ready, the buffer was removed by centrifugation at 1500× *g* for 2 min for the Zeba columns and 1000 x g for 4 min for the Bio-Spin columns at room temperature prior to analysis.

Analytical Methods

RP-HPLC-UV-MS

rhIGF-1/rhIGFBP-3 with Oxidized rhIGF-1/rhIGFBP-3 Spike-in Positive Control. Initial feasibility analysis of the rhIGF-1/rhIGFBP-3 control and rhIGF-1/rhIGFBP-3-furosemide admixture revealed small percentages of oxidized species present in low concentrations. To assess the method's ability to quantify the amounts of oxidized rhIGF-1 (oxi-rhIGF-1) and rhIGFBP-3 (oxi-rhIGFBP-3), a chemically oxidized rhIGF-1/rhIGFBP-3 (hydrogen peroxide-induced oxidation) was prepared, where the rhIGF-1/rhIGFBP-3 was exposed to a final hydrogen peroxide concentration of 0.005% (v/v), and used as a positive control. The chemically oxidized rhIGF-1/rhIGFBP-3 was spiked into a 50 μg/mL solution of rhIGF-1/rhIGFBP-3 at 0%, 1%, 2%, 5%, 10%, 20%, and 100% levels. A 26 μL aliquot of each of the spiked solutions was diluted to 13 μg/mL by adding 74 μL of rhIGF-1/rhIGFBP-3 formulation buffer to represent the mixing volumes followed by mixing with 15 μL of organic mixture solution and 1.15 μL of 10% triflouroacetic acid (TFA).

Admixture Sample Preparation with Zeba Column. Buffer exchange by Zeba column was conducted on 115 μL of the rhIGF-1/rhIGFBP-3-drug admixtures and control samples, which were transferred to the conditioned Zeba column. Centrifugation at 1500× *g* for 2 min was used to collect the sample from each Zeba column, to which 0.5 μL of 1% polysorbate 20 (PS20) and 1.2 μL of 10% TFA were added. Each buffer exchanged sample was transferred to an HPLC vial for RP-HPLC–UV–MS analysis.

RP-HPLC-UV-MS Setup and Parameters. A Thermo Scientific™ UltiMate™ HPG-3400RS Rapid Separation Binary pump (Mountain View, CA) with a Thermo Scientific™ Dionex™ UltiMate™ 3000 Diode Array Detector (DAD-3000) coupled online to a Thermo Q-Exactive HF mass spectrometer (San Jose, CA) was used for analysis. An Agilent Pursuit 3 Diphenyl analytical column (3.0 μm particle size, 2.0 mm i.d. × 150 mm, P/N A3041150 × 020) with a Thermo MabPac RP guard column (4.0 μm particle size, 2.1 mm i.d. × 10 mm, P/N 088649) was used for LC separation. A 6–port switching valve was used between the analytical and guard columns for desalting purposes. The injection volume was 80 μL (~0.25–2.0 μg injection amount per sample). Mobile phase A is 0.05% TFA in water and mobile phase B is 0.04% TFA in 75% ACN, 20% IPA, and 5% water. The LC column was equilibrated at 10% mobile phase B for 1 min prior to the gradient, then a curved gradient of 10% to 38% B over 30 min was used to elute the rhIGF-1/rhIGFBP-3 components for UV (214 nm) and MS detection.

RP-HPLC-UV-MS Data Analysis. Mass spectra of the rhIGF–1, rhIGFBP–3, and related species were assigned by matching masses deconvoluted by Biopharma Finder 2.0 (Thermo Fisher Scientific) to obtain neutral masses. The rhIGF-1 and rhIGFBP-3 related species were assigned by matching their observed mass to their corresponding theoretical mass (within 20 ppm relative to monoisotopic mass for rhIGF-1 and within 3 Da relative to average mass for rhIGFBP-3). The rhIGF-1 and rhIGFBP-3 related species were quantified using peak areas (extracted ion chromatogram, XIC) of the most intense isotopes from the most intense charge state for the desired species in the mass spectra. The percent oxidation was calculated from the peak area of the oxi–rhIGF–1 or oxi-rhIGFBP-3 species divided by the sum of the peak areas for both the unmodified and oxidized species. The equation for the percent oxi-rhIGF-1 calculation is shown below as an example.

$$\%\ oxi-rhIGF\text{-}1 = \left(\frac{Peak\ area\ oxi-rhIGF\text{-}1}{Peak\ areas(oxi-rhIGF\text{-}1+rhIGF\text{-}1)}\right)\times 100$$

The percent mass recovery of rhIGF-1/rhIGFBP-3 admixtures was calculated using the peak area from rhIGF-1/rhIGFBP-3 in its formulation buffer (unmodified control) as the 100% mass recovery control. The percent mass recoveries were calculated individually for both rhIGF–1 and rhIGFBP–3 using the sum peak areas of the unmodified and modified (oxidized) species as measured for the admixture relative to the same measurement for the 100% mass recovery control. The equation for total rhIGF-1 mass recovery calculation is shown below as an example.

$$\%\,rhIGF\text{-}1\ \text{recovery} = \left(\frac{\text{Sum of peak areas of oxi} - rhIGF\text{-}1\ \text{and}\ rhIGF\text{-}1\ \text{from}\ ''Admixture''}{\text{Sum of peak areas of oxi} - rhIGF\text{-}1\ \text{and}\ rhIGF\text{-}1\ \text{from}\ ''rhIGF\text{-}1/rhIGFBP-3\ \text{in formulation Buffer}''}\right)\times 100$$

Additionally, the oxidation value and the STDEV of the rhIGF-1/rhIGFBP-3 post mixing was compared to the controls; if there were no changes in this value or other attributes, such as increase in the high molecule weight formation (aggregation as assessed by SEC) or physical changes, the medication was deemed compatible. However, if there were any changes in any of the quality attributes, the medication was considered not compatible.

Potential adducts between the biologic components (rhIGF–1 or rhIGFBP–3) and small-molecule drug and/or excipients, if any, were first evaluated by checking the deconvoluted mass spectra for any unknown peaks, and then further assessed by evaluating the mass difference between the biologic components (rhIGF–1, rhIGFBP–3, oxidized and fragmented species) and corresponding unknown peaks. The identification was also confirmed by visual inspection of the raw mass spectra for the presence of actual adduct peaks (i.e. to avoid missed observations due to potential deconvolution artifacts).

SEC-UV-MS

SEC-UV–MS methods were developed to quantitate, at low concentrations, the main peak of the rhIGF-1/rhIGFBP-3 [intact protein complex (1:1 molar ratio of rhIGF–1:rhIGFBP–3)] and related HMW species (biologic aggregates) before and after mixing with small-molecule neonatal medications as well as any potential unbound rhIGF–1 or rhIGFBP–3. A rhIGF-1/rhIGFBP-3 admixture with caffeine citrate was used for method development and assessment of accuracy, LOD, LOQ, and mass recovery of main and HMW species; the HMW formation is expected if aggregation occurs.

rhIGF-1/rhIGFBP-3 Spiked with Forced Aggregated rhIGF-1/rhIGFBP-3 Positive Control. A forced aggregated rhIGF-1/rhIGFBP-3 drug substance standard was generated by thermally stressing (~5 hours at 60°C) of a drug substance lot followed by 20 minutes of vigorous vortexing at the highest speed. This sample served as a positive control for HMW species to quantitate the aggregate level (%), to determine the LOQ for rhIGF-1/rhIGFBP-3 aggregates and main peaks by UV and MS-XIC, and to evaluate the repeatability (triplicate analysis), relative standard deviation (RSD) and the percent spike recovery for total mass of rhIGF-1/rhIGFBP-3.

The rhIGF-1/rhIGFBP-3 plus rhIGF-1/rhIGFBP-3 forced aggregate mixture was spiked into the caffeine citrate admixture at a series of different ratios (0, 20, 40, 80, and 100% of forced aggregate levels) in which the total biologic concentration remained constant (5 μg/mL drug product), prepared in triplicate, held at room temperature for 30 min, and processed by Bio-Spin Column prior to analysis by SEC-UV-MS.

Admixture Analysis Preparation. Buffer exchange by Bio-Spin columns was conducted on 100 μL of the rhIGF-1/rhIGFBP-3-caffeine citrate admixture and control samples, which were mixed with 0.5 μL of a surfactant and subsequently transferred to the conditioned Bio-Spin Column. Centrifugation at 1000× *g* for 4 min was performed to collect the sample from each Bio-Spin Column. An additional 0.5 μL of 1% PS20 was added to each buffer–exchanged sample and transferred to HPLC vials for the SEC–UV–MS analysis.

SEC-UV-MS Setup and Parameters. A Thermo Scientific™ Vanquish™ UHPLC with a Thermo Scientific™ Vanquish Variable Wavelength Detector was coupled to a Thermo Q-Exactive BioPharma mass spectrometer (San Jose, CA) for the SEC-UV-MS setup. SEC separation was achieved using a Waters ACQUITY UPLC BEH125 size-exclusion column (1.7 μm particle size, 4.6 mm i.d. × 150 mm, P/N 186006505), with 50 mM ammonium acetate, pH 5.0 as the mobile phase (isocratic mode). The column temperature was set at 25 °C. An aliquot of 45 μL was loaded (~0.14 μg to 2.25 μg injection amount per sample) onto the column, followed by separation for 8 min with isocratic flow to elute the sample into the MS after which the SEC flow was diverted to waste through a 6-port switching valve to avoid introducing salt to the MS. The MS parameters used for analyzing rhIGF–1 and rhIGFBP–3 related species (HMW and Complex peaks) were the following: high mass range mode scan (m/z 2000–6000) with a mass resolution of 17,500, in–source CID of 30 eV, 3 microscans, AGC target of 3e6 with auto maximum ion injection time. For the UV setting, the wavelength was set at 210 nm.

SEC-UV-MS Data Analysis. The rhIGF-1/rhIGFBP-3 intact complex (1:1 rhIGF–1:rhIGFBP–3) and HMW species were assigned by the observed mass (deconvoluted by Biopharma Finder 3.0, Thermo Fisher Scientific), matching to their corresponding theoretical mass (within 1 Da relative to average mass for HMW and Complex peaks). The HMW and Complex peaks were quantified using peak area analysis from the XIC of the most intense isotopes from the top 5 most intense charge states for the desired species in the MS.

Results

RP-HPLC-UV-MS

Method Establishment

An LC-UV-MS method with Zeba column sample processing was first developed using the rhIGF-1/rhIGFBP-3-furosemide admixture to achieve sensitive detection of rhIGF-1, rhIGFBP-3, and corresponding oxidized counterparts when mixtures were at a clinically relevant concentration level (**Fig. 3**). A forced oxidized rhIGF-1/rhIGFBP-3 material (Oxidized Material) was then prepared and spiked into the furosemide admixture (at 0%, 1%, 2%, 5%, 10%, 20%, and 100% levels) to generate a standard curve and establish the accuracy of quantifying the oxi-IGF-1 and oxi-IGFBP-3 components (**Table S3**). As shown in **Fig. 3**, with a mass difference of one oxygen atom (i.e., 15.99 Da) between rhIGF–1 and oxi–rhIGF–1, and the oxidized form being chromatographically resolved from the unmodified form, the level of rhIGF-1 oxidation was precisely determined using LC–MS (XIC) with good linearity (LOD; R^2=1.00) and high reproducibility (RSD ≤3.0 %) obtained for all the spiked in levels of oxidized material. The LOQ was determined to be 1.6% oxi-rhIGF-1. The oxi–rhIGFBP–3 closely eluted with the unmodified rhIGFBP–3 but was precisely determined using LC-MS (XIC), with good linearity (LOD; R^2=0.99) and high reproducibility (RSD <10.0 %) obtained for all the spiked levels tested. The LOQ was determined to be 0.13% oxi-rhIGFBP-3.

Assessment of Method Performance – rhIGF-1/rhIGFBP-3 Admixtures with Eight Neonatal Drugs

The performance and the general applicability of the developed method to other admixtures was systematically studied and confirmed with an additional eight admixtures including caffeine citrate,

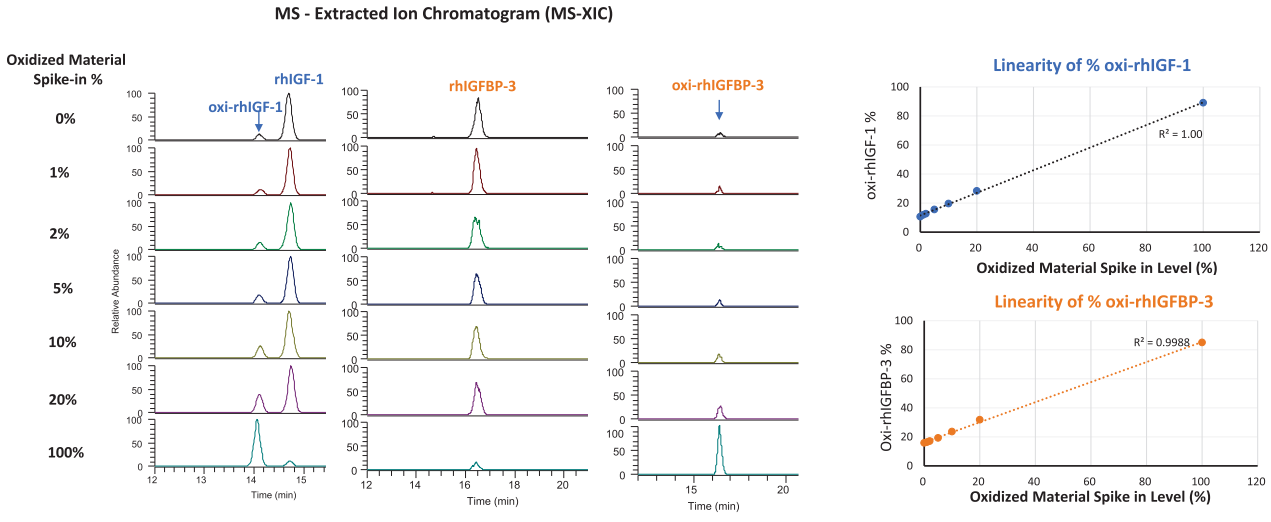


Figure 3. Quantitation of oxi-rhIGF-1 and oxi-rhIGFBP-3 in rhIGF-1/rhIGFBP-3-furosemide admixture using RP-UV-MS (XIC). XIC is based on the m/z of the highest isotope of the most abundant charge state. rh, recombinant human; RP-UV-MS, reversed-phase ultraviolet with mass spectrometric detection; XIC, extracted ion chromatogram.

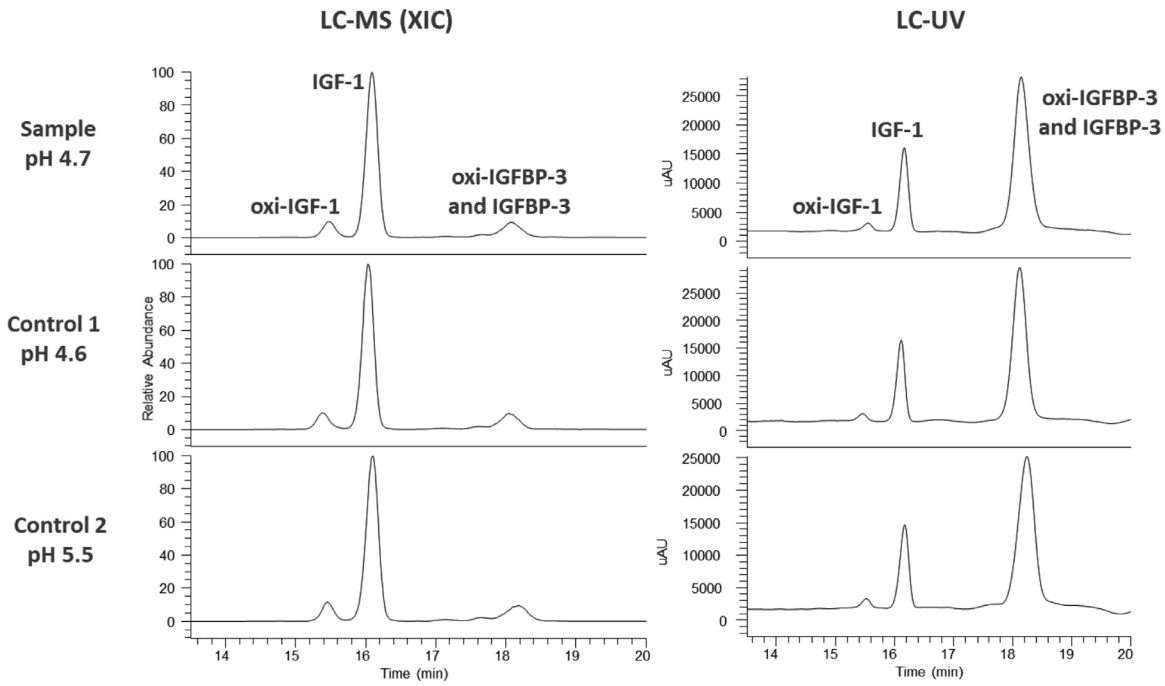


Figure 4. Quantitation of oxidation levels in rhIGF-1/rhIGFBP-3-caffeine citrate admixture using RP-HPLC-MS (XIC) and RP-HPLC-UV. XIC is based on the m/z of the highest isotope of the most abundant charge state. RP-HPLC-MS, reversed-phase high-performance liquid chromatography with mass spectrometric detection; RP-HPLC-UV-MS, reversed-phase high-performance liquid chromatography ultraviolet with mass spectrometric detection; XIC, extracted ion chromatogram.

ampicillin, vancomycin, penicillin G, gentamicin, hydrocortisone, cefotaxime, and dopamine.[1] This set of medications was selected from multiple therapeutic categories (Fig. 1), with varying chemical structures, formulation excipients, and a wide range of final post-mixing pH values (~4.0 to ~9.0) (**Table S1**). Reproducible and accurate quantitation of the percent level of oxi-IGF-1 and oxi-IGFBP-3 were obtained for all tested admixtures. Among these and as an example, the rhIGF-1/rhIGFBP-3-caffeine citrate admixture had similar oxidation levels to those in the corresponding control samples, diluted in either the caffeine citrate mixture or with formulation buffer (Fig. 4). The percent aggregation level (HMW species), as assessed by SEC-UV-MS, did not increase due to admixing (1.4% HMW species for the admixture vs 1.3% and 1.5% for the caffeine citrate or the formulation buffer diluted controls). Post-mixing degradation levels for the rhIGF-1/rhIGFBP-3-caffeine citrate admixture were assessed by RP-HPLC-UV-MS (Fig. 5**A**). Across the admixture sample and Controls 1 and 2 (**Table S2**), % oxi-rhIGF-1 ranged from 9.0 ± 0.2 for the admixture sample (pH 4.7) to 9.3 ± 0.4 for the pH unadjusted Control 2 (pH 5.5), while the percent oxi-rhIGFBP-3 ranged from 10.9 ± 0.0 for Control 2 to 11.8 ± 0.3 for the pH-adjusted (pH 4.6) Control 1. Two rhIGFBP-3 fragments were quantified; the F1-rhIGHBP-3 ranged from 4.3% to 5.4% while the F2-rhIGFBP-3 ranged from 1.2% to 1.3%, which did not change for the admixture samples when compared with the control solutions. Overall, no degradation was observed and the total mass recovery of rhIGF-1/rhIGFBP-3 was high (>90% for both IGF-1 and IGFBP-3 species) for the caffeine citrate admixture and controls.

As another example, the rIGF-1/rhIGFBP-3-ampicillin admixture post-mixing degradation levels, assessed by LC-UV-MS, are shown in Fig. 5**B**. Oxidation levels in the admixture (Table 1) were similar to those in the control samples of rhIGF-1/rhIGFBP-3 alone, suggesting that oxidation levels did not increase after mixing. There was a lower mass recovery of rhIGF-1/rhIGFBP-3 in the admixture as compared with controls. An ampicillin-rhIGFBP-3 adduct was observed in the MS spectra, which likely contributed to the lower mass recovery observed. The ampicillin adduct was also confirmed by the SEC method (Fig. 5**C**) but indicated that ampicillin admixing did not cause additional aggregation of rhIGF-1/rhIGFBP-3.

The penicillin G admixture showed a similar behavior with no increase in the oxidation levels post mixing but had a lower mass recovery (70%), thus indicating a loss that may be attributed to adduct formation. The vancomycin admixture had no effect on oxidation or degradation. Vancomycin and penicillin G will further be assessed by the SEC methods.

HTP Analysis of rhIGF-1/rhIGFBP-3 Admixtures with Sixteen Commonly Used Neonatal Drugs

An additional 16 commonly used drugs were further studied using the developed RP-HPLC-UV-MS method. Based on the studies, admixtures of rhIGF-1/rhIGFBP-3 with dopamine (tested with rhIGF-1/rhIGFBP-3 drug product lots #1 and #2) and norepinephrine bitartrate (Levophed), but not epinephrine, caused oxidative degradation of the rhIGF-1/rhIGFBP-3 components (Table 1). Oxidation increased substantially for the dopamine and dobutamine admixtures (>~70% for both oxi-rhIGF-1 and oxi-rhIGFBP-3) as compared with controls. Oxidation increased by 2–fold for the norepinephrine admixture, with oxi–rhIGF–1 increasing from ~8–9% in control samples to ~15–16% in the admixture and with oxi–rhIGFBP–3 increasing from ~12–13% in controls to ~23–24% in the admixture. Increased oxidative degradation of rhIGF-1/rhIGFBP-3 is most likely due to the presence of the excipient bisulfite/metabisulfite, which is included in dopamine and dobutamine formulations. In addition to the increased oxidation, lower protein recoveries were observed for dopamine and dobutamine admixtures (~54–68% based on rhIGFBP-3, Table 1), as compared with norepinephrine and epinephrine (both >~90%). Admixtures of rhIGF-1/rhIGFBP-3 with dopamine, norepinephrine, epinephrine, and dobutamine did not cause additional fragmentation of rhIGFBP-3, which remained essentially unchanged (≤2% F2-rhIGFBP-3) for all tested admixtures and controls (data not shown).

[1] Dopamine was tested with two different lots of rhIGF-1/rhIGFBP-3 drug product; method performance was tested using lot #1.

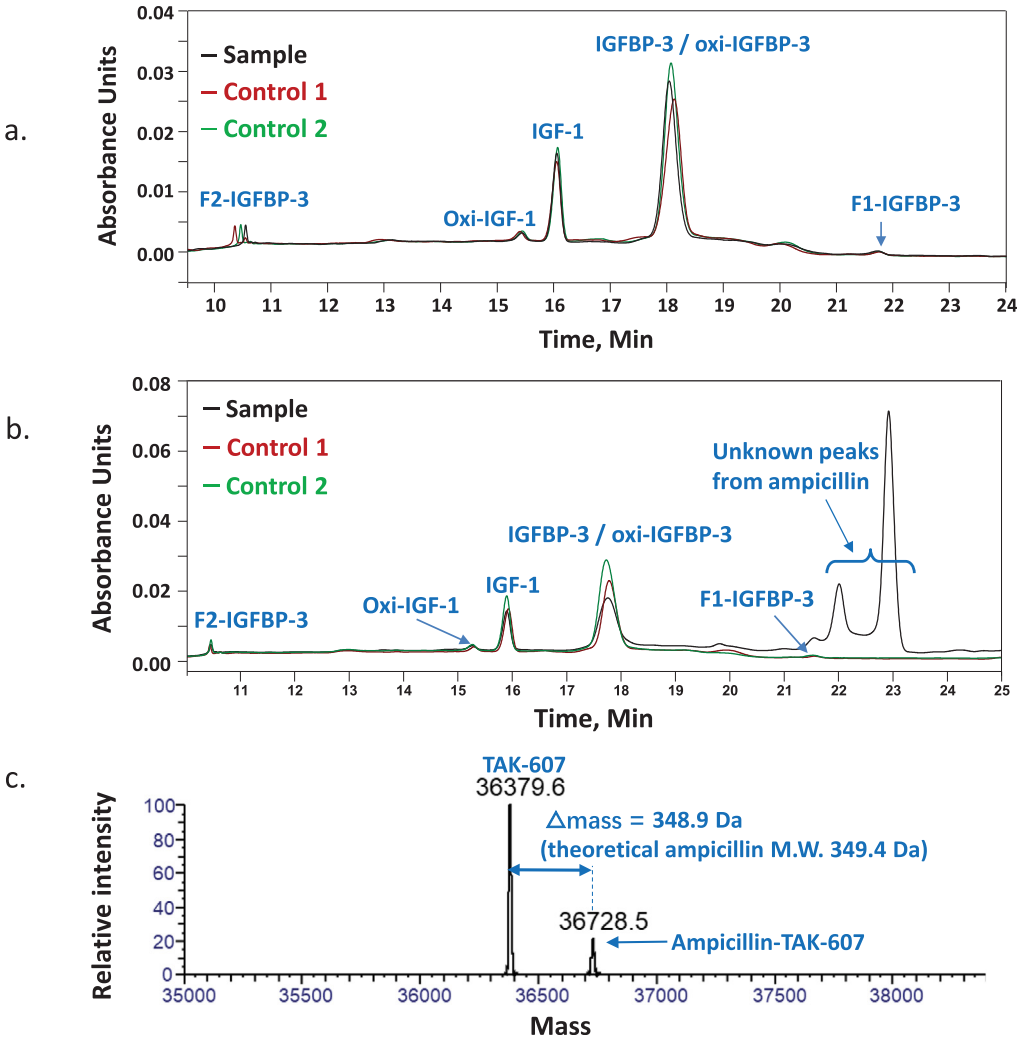


Figure 5. rhIGF-1/rhIGFBP-3 degradation levels assessed by RP-HPLC-UV (214 nm) in rhIGF-1/rhIGFBP-3 for panel a: caffeine citrate admixture and panel b: ampicillin admixture. Panel c: ampicillin-rhIGF-1/rhIGFBP-3 adduct observed by native SEC-MS. F1 and F2, fragments; oxi, oxidation product; rh, recombinant human; RP-HPLC-UV, reversed-phase high-performance liquid chromatography with ultraviolet detection. Control 1: rhIGF-1/rhIGFBP-3 drug product diluted and pH adjusted. Control 2: rhIGF-1/rhIGFBP-3 drug product diluted with the biologic formulation buffer.

For fluconazole sodium chloride, dexmedetomidine hydrochloride (Precedex), ceftazidime pentahydrate, and insulin (Novolin R), no oxidative degradation or fragmentation was seen for the admixtures; the protein recoveries were high (87–100%) for all admixtures and no adduct formation was detected.

For clonidine (Duraclon) and potassium canrenoate (Soldactone), admixtures with rhIGF-1/rhIGFBP-3 caused no additional oxidation. However, rhIGF-1/rhIGFBP-3 admixtures with menatetrenone (KayTwo) and acyclovir sodium caused notable increases in oxidation; an increase of 3–6% for oxi-rhIGF-1 and 9% for oxi-rhIGFBP-3 (**Table 1**). No additional fragmentation was seen for any of these admixtures. The protein recoveries were high (86–102%) for all admixtures and no adduct formation was detected.

For rocuronium, succinylcholine chloride (Anectine) and ranitidine, oxidative degradation or fragmentation was not observed in any of these admixtures. The protein recoveries were high for both rhIGF-1 and rhIGFBP-3 (87–100%) and no adducts were detected in these admixtures. For daptomycin admixture, a complete loss of rhIGF-1 and rhIGFBP-3 signals was observed. Further analyses of the admixture using different sample preparation procedures, or direct injection into the MS detector, did not produce a MS signal for either rhIGF-1/rhIGFBP-3 component. As daptomycin has been reported to have a high propensity for binding proteins (~92–94%),[10–12] the loss of rhIGF-1/rhIGFBP-3 signal post mixing is most likely attributable to product's reduced solubility owing to the small molecule's binding property (**Table 1**).

SEC-UV-MS Analysis of rhIGF-1/rhIGFBP-3 Admixture with Caffeine Citrate

An SEC-UV-MS method together with Bio-Spin sample processing was first developed using rhIGF-1/rhIGFBP-3-caffeine citrate admixture to achieve the sensitive detection of rhIGF-1/rhIGFBP-3 aggregates, intact biologic complex, and any unbound rhIGF-1 or rhIGFBP-3 at low concentration (**Fig. 6**). Similar to the approaches used for the RP-HPLC, method development of a forced aggregated rhIGF-1/rhIGFBP-3 material was then prepared and spiked into the caffeine admixture (at 0%, 10%, 20%, 40%, 80%, and 100% levels) to assess the method's ability to accurately quantify the level of HMW, intact biologic complex, and free species (**Fig. 7**). All species were well separated, with good linearity (LOD; R^2=0.99) and <2.0 % RSD for the intact rhIGF-1/rhIGFBP-3 and <10.0 % RSD for the HMW species for all spike levels tested. The LOQ for quantitation of percent HMW species in an admixture was ~0.5%. Admixing with caffeine citrate had

Table 1
RP-HPLC-UV-MS Admixture Compatibility Assessment Study Results: Analysis of Degradants,[1] Recoveries,1 and Compatibility. The First Two Rows Represent Examples of Controls, where the Data for the RhIGF-1/rhIGFBP-3 Drug Product Diluted in its Formulation Buffer (Control 1, 5 μg/mL DP) and when Diluted In a Matrix (Saline for this Example and in the Absence of a Small Molecule; Control 2, 49 μg/mL DP) are Shown.

Admixtures	Admixture pH	Degradants Level, Avg ± SD (n = 3), MS (XIC)		Total Mass Recovery		Compatibility Assessment
		% oxi-rhIGF-1	% oxi-rhIGFBP-3	rhIGF-1 Species	rhIGFBP-3 Species	
Control 1	5.5	9.3 ± 0.40	10.9 ± 0.60	100	100	n.a.
Control 2	5.48	8.88 ± 0.04	13.1 ± 0.16	97.24 ± 0.02	103.09 ± 0.06	n.a.
Furosemide	n.a.	10.7 ± 0.31	15.9 ± 1.10	**80.77 ± 0.67**	**78.18 ± 4.61**	N, based on physical studies
Caffeine Citrate	4.7	9.0 ± 0.15	11.4 ± 0.38	93.69 ± 6.14	90.99 ± 9.51	Y
Ampicillin monosodium salt	8.9	8.5 ± 0.09	11.7 ± 0.34	**71.36 ± 0.14**	**54.88 ± 0.44**	N, adduct formation
Vancomycin hydrochloride	4.2	8.5 ± 0.10	11.6 ± 0.10	91.00 ± 6.16	88.54 ± 8.88	Y
Penicillin G	5.6	8.6 ± 0.16	12.0 ± 0.52	**76.73 ± 1.25**	**67.04 ± 4.31**	N, adduct formation
Gentamicin	5.17	8.43 ± 0.07	11.67 ± 0.53	103.71 ± 2.33	94.66 ± 1.03	Y
Hydrocortisone sodium succinate	5.88	8.07 ± 0.05	12.37 ± 0.42	89.45 ± 1.42	93.26 ± 3.03	Y
Cefotaxime sodium	5.42	8.55 ± 0.11	11.84 ± 0.28	82.47 ± 1.53	**76.48 ± 5.45**	Y
Dopamine	5.21	**65.70 ± 8.92**[2]	**70.92 ± 3.84**[2]	100.32 ± 3.65	**68.04 ± 15.26**	N, oxidation
Norepinephrine bitartrate (Levophed)	5.44	**16.05 ± 0.16**[2]	**23.92 ± 0.29**[2]	89.42 ± 0.06	94.26 ± 0.04	N, oxidation
Epinephrine	5.5	8.68 ± 0.07[2]	13.47 ± 0.22[2]	92.95 ± 0.02	91.43 ± 0.06	Y
Dobutamine hydrochloride	5.4	**99.45 ± 0.1**[2]	**86.32 ± 2.62**[2]	90.91 ± 0.04	**53.97 ± 0.03**	N, oxidation
Fluconazole (Diflucan)	5.5	8.82 ± 0.15[2]	13.66 ± 0.04[2]	87.42 ± 0.02	89.49 ± 0	Y
Dexmedetomidine hydrochloride, (Precedex)	5.48	8.57 ± 0.21[2]	13.29 ± 0.18[2]	97.84 ± 0.01	94.94 ± 0.04	Y
Ceftazidime	6.81	8.48 ± 0.09[2]	13.34 ± 0.05[2]	95.58 ± 0.02	89.12 ± 0.01	Y
Insulin (Novolin R)	5.48	8.2 ± 0.05[2]	13.16 ± 0.1[2]	99.01 ± 0.03	102.75 ± 0.07	Y
Clonidine hydrochloride (Duraclon)	5.49	8.88 ± 0.05[2]	13.08 ± 0.05[2]	93.12 ± 0	101.89 ± 0.03	Y
Potassium Canrenoate (Soldactone)	7.01	7.85 ± 0.13[2]	13.39 ± 0.24[2]	87.59 ± 0.04	97.33 ± 0.07	Y
Menatetrenone (KayTwo)	6.81	11.04 ± 0.19[2]	**22.08 ± 1.15**[2]	**85.8 ± 0.06**	86.87 ± 0.13	N, oxidation
Acyclovir sodium	10.63	**14.9 ± 0.35**[2]	**22.20 ± 1.62**[2]	96.51 ± 0	91.75 ± 0.05	N, oxidation
Daptomycin[2]	4.84	ND[3]	ND[3]	<1%	<1%	N
Rocuronium	4.12	8.01 ± 0.04[2]	13.68 ± 0.15[2]	88.72 ± 0.06	93.14 ± 0.07	Y
Succinylcholine Chloride (Anectine)	4.58	8.57 ± 0.01[2]	13.81 ± 0.06[2]	96.66 ± 0.08	91.35 ± 0.12	Y
Ranitidine	6.55	8.09 ± 0.01[2]	13.29 ± 0.06[2]	93.46 ± 0.04	94.13 ± 0.02	Y

IGF-1, insulin-like growth factor-1; IGFBP-3, insulin-like growth factor binding protein-3; MS, mass spectrometry; ND, not detected; rh, recombinant human; RP-HPLC, reversed-phase high-performance liquid chromatography; SD, standard deviation; UV, ultraviolet; XIC, extracted ion chromatogram.

[1] Increased oxidation (>15% % oxi-rhIGF-1; >20% % oxi-rhIGFBP-3) or low total mass recovery (<~80% rhIGF-1 species; <~80% rhIGFBP-3 species) was observed for certain admixtures and those are shown in **bold** text above.

[2] For n = 2, SD equals 0.7071 times the range.

minimal effect on the percent of HMW species. The LOD / LOQ for free IGF-1 in admixture was 1 μg/mL, and LOD / LOQ for free IGFBP-3 species in admixture were 0.5 and 1 μg/mL, respectively.

Discussion

Premature infants in the neonatal intensive care unit often require simultaneous administration of multiple parenteral medications with limited intravenous access. However, at present, little is known about the compatibility of drugs currently in development with medications used in standard practice.[13] Increased risk of formation of visible particles, precipitation, and other physical changes that may indicate significant chemical changes are known effects with some combinations. For example, literature cites that furosemide (10 mg/mL) and midazolam (1 mg/mL) co-administered at the same infusion rate (2 mg/h) resulted in 10-15% furosemide drug loss,[14] fatal

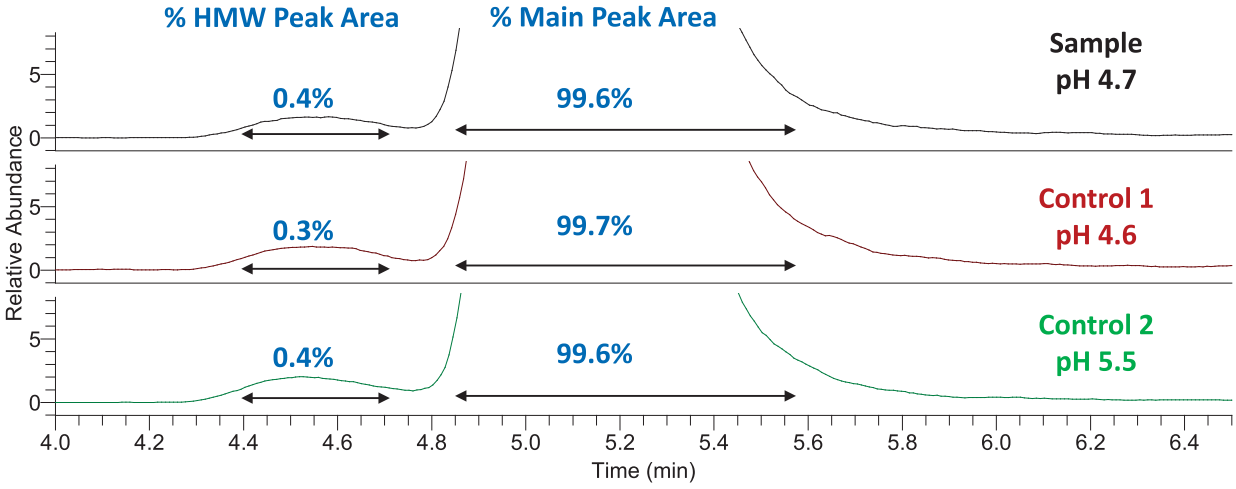


Figure 6. Aggregation (HMW) levels in rhIGF-1/rhIGFBP-3 and caffeine citrate admixture by SEC-MS (XIC). XIC is based on the m/z of the highest isotopes of the top 5 charge states. HMW, high molecular weight; MS, mass spectrometry; rh, recombinant human; SEC-UV, size-exclusion ultra-performance liquid chromatography with ultraviolet detection; XIC, extracted ion chromatogram. Control 1: rhIGF-1/rhIGFBP-3 drug product diluted with caffeine citrate matrix. Control 2: rhIGF-1/rhIGFBP-3 diluted with the biologic formulation buffer.

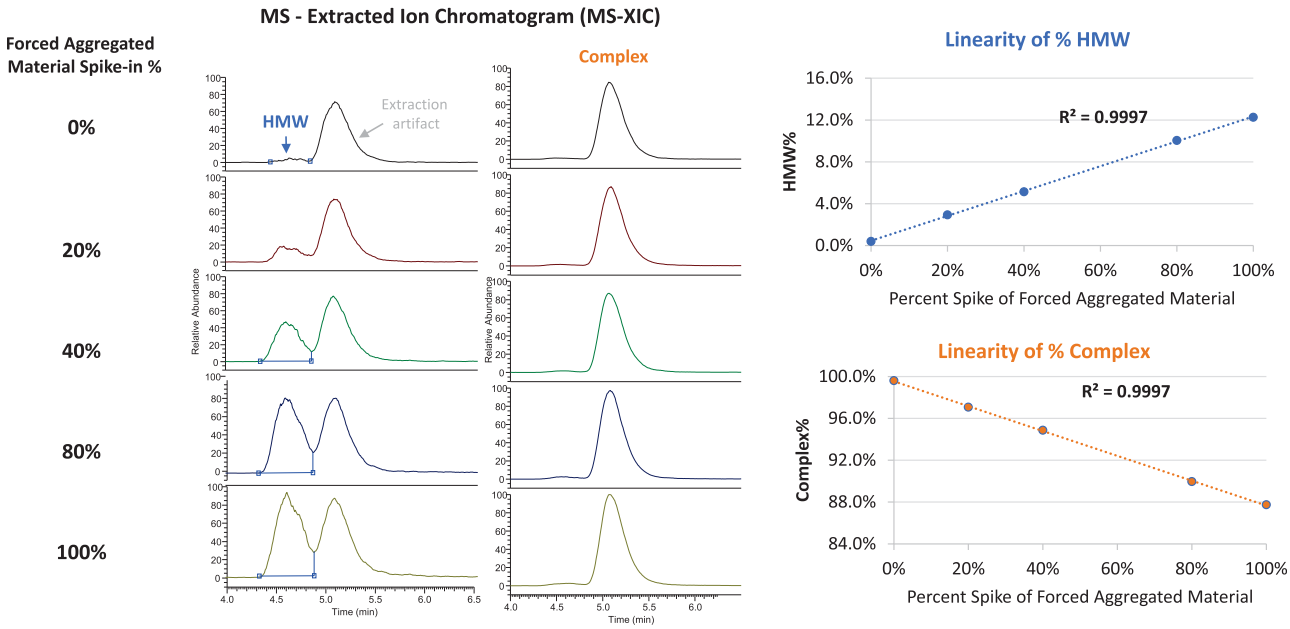


Figure 7. Quantitation of HMW and intact rhIGF-1/rhIGFBP-3 complex in rhIGF-1/rhIGFBP-3-caffeine citrate admixture by SEC-UV-MS (XIC). XIC is based on the m/z of the highest isotopes of the top 5 charge states. HMW, high molecular weight; SEC-UV-MS, size-exclusion ultra-performance liquid chromatography; XIC, extracted ion chromatogram.

cardiopulmonary complications due to precipitation between ceftriaxone and calcium electrolyte solutions have also been documented in neonates,[15] and semisynthetic penicillin complexation with aminoglycosides has been shown to result in a loss of antibiotic activity.[16] However, few combinations of medications with biologics have been evaluated to date.[13]

To address such concerns, we developed a high-thoughput and sensitive assay to test the compatibility and detect any potential molecular changes of rhIGF-1/rhIGFBP-3 and its degradants (oxidized species, potential fragments, and formation of HMW species), at ultra low concentrations, with medications often administered to extremely premature newborns using RPLC-UV-MS and SEC-UV-MS. These two methods were used for testing of mixtures of rhIGF-1/rhIGFBP-3 with a panel of small-molecule drugs. In these studies, the rhIGF-1/rhIGFBP-3 concentration range was qualified in selected admixtures and rhIGF-1/rhIGFBP-3 components and degradants in the mixture were accurately quantified.

The RPLC-UV-MS method was optimized to investigate the impact admixtures of furosemide, caffeine citrate, and ampicillin, as three example drugs commonly prescribed to neonates on rhIGF-1/rhIGFBP-3. Findings from this study revealed that, although furosemide formulation resulted in an increased pH value as compared to the control value, no increased degradation of rhIGF-1/rhIGFBP-3 components was observed in the admixture (only assessed by RP-HPLC); however, the product is not compatible with rhIGF-1/rhIGFBP-3 as physical studies demonstrated turbid solutions post mixing. Admixtures of rhIGF-1/rhIGFBP-3 with ampicillin or penicillin G did not result in rhIGF-1/rhIGFBP-3 oxidation or fragmentation; however, there was lower mass recovery of the rhIGF-1/rhIGFBP-3 components with no visual turbidity, which likely came from ampicillin- or penicillin G-rhIGFBP-3 adducts detected post mixing. After admixing rhIGF-1/rhIGFBP-3 with caffeine citrate, no increase in oxidation, fragmentation, aggregation, or increase in free sub-molecular species levels was observed using this methodology, and there was acceptable mass recovery of rhIGF-1/rhIGFBP-3 drug product. Analysis of serial dilutions of rhIGF-1/rhIGFBP-3 in mixtures with caffeine citrate or ampicillin resulted in highly reproducible and linear results at clinically relevant low concentration ranges, consistent with results from corresponding pH-matched and protein-concentration controls. These results also demonstrate that mixing and/or pH change does not necessarily result in further oxidation or fragmentation of the drug product; however, further analyses may be needed to confirm the suitability of mixtures for administration. These preliminary findings from RP-HPLC-MS as well as SEC-UPLC-UV and SEC-HPLC-MS provide support for the co-administration of rhIGF-1/rhIGFBP-3 with caffeine citrate. However, the initial findings demonstrate a potential risk for mixing rhIGF-1/rhIGFBP-3 with ampicillin and penicillin G that warrants further investigation. Ampicillin and penicillin G belong to a class of antibiotics that have been reported to react with the lysine side chains of protein drugs,[17] which in this study likely affected the related mass recovery. The stability of these aminopenicillins appears to be dependent on both pH and temperature of the aqueous solution, which may result in a loss of their antibiotic effect at alkaline pH values.[18,19] rhIGF-1/rhIGFBP-3 admixtures with gentamicin, hydrocortisone, and cefotaxime showed no oxidation or fragmentation; however, there was lower mass recovery of the rhIGFBP-3 component for cefotaxime (76.5%) which was not associated with adduct formation that requires further investigation for characterization. Additionally, we found that dopamine, norepinephrine bitartrate (Levophed), dobutamine, menatetrenone (KayTwo, a derivative of vitamin K), and acyclovir produced significant oxidation of rhIGF-1 and rhIGFBP-3 components upon admixing, which resulted in reduced recoveries. Thus, by these studies, these compounds are determined to be noncompatible for central line Y-site IV co-administration with rhIGF-1/rhIGFBP-3.

In their 2010 review of neonatal drug studies, Kalikstad et al. report that for almost 60% of IV drug–drug infusions, there is no compatibility documentation.[5] Physical compatibility is required as a minimum for co-infusion of two drugs through a Y-site IV line as the consequences of co-infusing incompatible drugs could contribute to unsafe patient treatment practices.[6] Physical compatibility is usually defined as no occurrence of precipitation, change in color or pH, the appearance of particulates, or production of gas.[6,8] While many of the drug combinations commonly used in neonates show no physical incompatibilities,[7,8] tests for physical compatibility are usually tested by eye and do not necessarily rule out chemical incompatibility, particularly when the drug is present at very low concentration.[6,7] Chemical compatibility of biologics should also be assessed but

requires different analyses from those carried out in the physical compatibility studies. Physical changes may or may not be closely associated with chemical changes, and therefore tests for chemical compatibility cannot be inferred via physical compatibility studies except when instances are extreme and obvious.[6]

The totality of findings from this study supports the use of these methodologies for detecting protein modifications under the clinical administration scenarios and for supplementing the risk assessment available from physical compatibility studies. Our data add to the limited data available on chemical compatibility of IV biologic drugs co-administered to preterm infants.

Study Limitations

Previous studies suggest that covalent modifications are often associated with the beta-lactam reactions of penicillin with proteins resulting in immunogenicity.[17,20,21] Little is known about specific penicillin adduct formation identified in the study, and further studies may be needed to identify the specific nature of the observed ampicillin- and penicillin G-rhIGFBP-3 adducts, as no direct structural information can be discerned from intact MS and UV spectra. Additionally, a cell-based potency assay may be needed to provide further understanding on when additional factors should be considered in risk assessments of admixed drugs. Furthermore, although authors routinely study the subvisible particles for the product itself, based on the development knowledge of the product, the formation / increase in number of subvisible particles is not expected to be an additional degradation route for the product.

Lastly, it will be ideal to potentially mimic the mixing occurring in the actual adminstration sets; however, any physical incompatibility observations will be obscured by lack of transparent components. With the established methods, separate studies may be conducted to study the compabtibility of the admixtures with the administration components.

Conclusion

Protein-specific methodologies have been developed to test compatibility of rhIGF-1/rhIGFBP-3 and common medications co-administered to neonates within therapeutic ranges anticipated for treatment of extremely premature newborns, and at levels consistent with dosing in a clinical trial. Detection of ultra-low concentrations of rhIGF-1/rhIGFBP-3 drug product with caffeine citrate and ampicillin, among other neonatal IV medications, was demonstrated. Our findings suggest that co-administration of rhIGF-1/rhIGFBP-3 with caffeine citrate poses low risk to drug safety and efficacy; however, further investigation is recommended before co-administering with ampicillin and other potentially incompatible medications. Our methods augment routine physical compatibility assessments and inform the potential of chemical changes to the rhIGF-1/rhIGFBP-3 complex. Further, we establish methods to evaluate the degradation route of the rhIGF-1/rhIGFBP-3 drug product upon admixing with other medicines. These findings elucidated by new analytical methods, as reported here, support a risk-based approach for assessing compatibility of biologic admixtures in general and specifically for commonly used drugs in neonates.

Data Availability

Datasets are available upon reasonable request. Data will be made available after publication of the study results within 3 months from initial request, to researchers who provide a methodologically sound proposal.

Conflicts of Interest

- Mark Turner's employing institution has a consultancy agreement with Takeda relating to his involvement with this product.
- Christopher McPherson has a consultancy agreement with Shire PLC, a member of the Takeda group of companies, relating to this product.
- Dongdong Wang, Norman Barton, Paul A. Salinas, and Nazila Salamat-Miller, are employees of Takeda, and own stock/stock options in Takeda.
- Jennifer S. Chadwick and Wanlu Qu are employees of BioAnalytix, Inc., and were paid consultants to Shire PLC, a member of the Takeda group of companies, in relation to this study. During this study, Dongdong Wang was an employee of BioAnalytix, Inc.
- This study was funded by Shire, a Takeda company. Funding for medical writing assistance was also provided by Takeda.

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Supplementary Materials

Supplementary material associated with this article can be found in the online version at doi:10.1016/j.xphs.2021.10.027.

References

1. Hellstrom A, Ley D, Hallberg B, et al. IGF-1 as a drug for preterm infants: a stepwise clinical development. *Curr Pharm Des*. 2017;23(38):5964–5970.
2. Hellstrom A, Engstrom E, Hard AL, et al. Postnatal serum insulin-like growth factor I deficiency is associated with retinopathy of prematurity and other complications of premature birth. *Pediatrics*. 2003;112(5):1016–1020.
3. Hansen-Pupp I, Hellstrom A, Hamdani M, et al. Continuous longitudinal infusion of rhIGF-1/rhIGFBP-3 in extremely preterm infants: evaluation of feasibility in a phase II study. *Growth Horm IGF Res*. 2017;36:44–51.
4. Ley D, Hallberg B, Hansen-Pupp I, et al. rhIGF-1/rhIGFBP-3 in preterm infants: a phase 2 randomized controlled trial. *J Pediatr*. 2019;206:56.e8–65.e8.
5. Kalikstad B, Skjerdal A, Hansen TW. Compatibility of drug infusions in the NICU. *Arch Dis Child*. 2010;95(9):745–748.
6. Kanji S, Lam J, Johanson C, et al. Systematic review of physical and chemical compatibility of commonly used medications administered by continuous infusion in intensive care units. *Crit Care Med*. 2010;38(9):1890–1898.
7. Fox LM, Wilder AG, Foushee JA. Physical compatibility of various drugs with neonatal total parenteral nutrient solution during simulated Y-site administration. *Am J Health Syst Pharm*. 2013;70(6):520–524.
8. Yamashita SK, Walker SE, Choudhury T, Iazzetta J. Compatibility of selected critical care drugs during simulated Y-site administration. *Am J Health Syst Pharm*. 1996;53(9):1048–1051.
9. Baririan N, Chanteux H, Viaene E, Servais H, Tulkens PM. Stability and compatibility study of cefepime in comparison with ceftazidime for potential administration by continuous infusion under conditions pertinent to ambulatory treatment of cystic fibrosis patients and to administration in intensive care units. *J Antimicrob Chemother*. 2003;51(3):651–658.
10. FDA. CUBICIN® (daptomycin for injection). Highlights of Prescribing Information. 2003. Available at: https://www.accessdata.fda.gov/drugsatfda_docs/label/2011/021572s038lbl.pdf. Accessed November 8, 2021.
11. Garrison MW, Vance-Bryan K, Larson TA, Toscano JP, Rotschafer JC. Assessment of effects of protein binding on daptomycin and vancomycin killing of Staphylococcus aureus by using an in vitro pharmacodynamic model. *Antimicrob Agents Chemother*. 1990;34(10):1925–1931.
12. Dvorchik BH, Brazier D, DeBruin MF, Arbeit RD. Daptomycin pharmacokinetics and safety following administration of escalating doses once daily to healthy subjects. *Antimicrob Agents Chemother*. 2003;47(4):1318–1323.
13. Girardi A, Galletti S, Raschi E, et al. Pattern of drug use among preterm neonates: results from an Italian neonatal intensive care unit. *Ital J Pediatr*. 2017;43(1):37.

14. Foinard A, Decaudin B, Barthelemy C, Debaene B, Odou P. Impact of physical incompatibility on drug mass flow rates: example of furosemide-midazolam incompatibility. *Ann Intensive Care*. 2012;2(1):28.
15. Bradley JS, Wassel RT, Lee L, Nambiar S. Intravenous ceftriaxone and calcium in the neonate: assessing the risk for cardiopulmonary adverse events. *Pediatrics*. 2009;123(4):e609–e613.
16. Pickering LK, Rutherford I. Effect of concentration and time upon inactivation of tobramycin, gentamicin, netilmicin and amikacin by azlocillin, carbenicillin, mecillinam, mezlocillin and piperacillin. *J Pharmacol Exp Ther*. 1981;217(2):345–349.
17. Corran PH, Waley SG. The reaction of penicillin with proteins. *Biochem J*. 1975;149(2):357–364.
18. Bundgaard H, Hansen J. Reaction of ampicillin with serum albumin to produce penicilloyl-protein conjugates and a piperazinedione. *J Pharm Pharmacol*. 1982;34(5):304–309.
19. do Nascimento TG, de Jesus Oliveira E, Basilio Junior ID, de Araujo-Junior JX, Macedo RO. Short-term stability studies of ampicillin and cephalexin in aqueous solution and human plasma: application of least squares method in Arrhenius equation. *J Pharm Biomed Anal*. 2013;73:59–64.
20. Tsuji A, Yamana T, Miyamoto E, Kiya E. Chemical reactions involved in penicillin allergy: kinetics and mechanism of penicillin aminolysis. *J Pharm Pharmacol*. 1975;27(8):580–587.
21. Weltzien HU, Padovan E. Molecular features of penicillin allergy. *J Invest Dermatol*. 1998;110(3):203–206.