Journal of Pharmaceutical Sciences 110 (2021) 3268–3275

Contents lists available at ScienceDirect

Journal of Pharmaceutical Sciences

journal homepage: www.jpharmsci.org

Pharmaceutics, Drug Delivery and Pharmaceutical Technology

4-Hydroxynonenal – A Toxic Leachable from Clinically Used Administration Materials

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ARTICLE INFO

Article history:
Received 8 February 2021
Revised 28 May 2021
Accepted 28 May 2021
Available online 4 June 2021

Keywords:
Adducts
Degradation product
Injectable(s)
Lipids
Liquid chromatography-mass spectrometry (LC-MS)
Oxidation
Polymers
Proteins
Surfactants
Toxicity

ABSTRACT

Introduction: The migration of chemicals from processing materials into biopharmaceuticals can lead to various problems. Leachables from administration materials, with no possibility of further clearance, are of particular concern. Released chemicals can be toxic or react with formulation components, thereby impacting product safety. Therapeutic proteins, which are susceptible to chemical modifications, have highest risk to be affected.
Aim: The aim of this study was to identify a previously unknown leachable compound from clinical administration sets, which was present above the applied generic safety threshold.
Methods: Extracts of commonly used clinical administration sets were analyzed using a recently established specific assay allowing the identification and quantification of the α,β-unsaturated aldehyde 4-hydroxynonenal (HNE) in a drug product surrogate solution. HNE was quantified after derivatization with 2,4-dinitrophenylhydrazine (DNPH) and liquid extraction of the formed hydrazone by LC-MRM analysis.
Results: Potentially genotoxic HNE was a leachable compound from all tested administration sets, in parts exceeding safety thresholds for genotoxicants. The HNE-releasing polymer was identified as PVC.
Conclusion: Clinical administration sets should be, like manufacturing materials and container closure systems, in the focus of routine leachables studies. Manufacturers of clinical administration sets should show responsibility to avoid the presence of safety concerning chemicals, like HNE.

Introduction

During the complex process of biopharmaceuticals' manufacturing, storage and administration the drug products (DPs) and their constituents are in contact with various material surfaces, increasingly composed of plastics. It should be noted that this also concerns DPs containing small molecules as active pharmaceutical ingredients. It is well known that chemical compounds originating from the plastic materials are able to migrate during such contacts into the formulation containing the active pharmaceutical ingredient (API).[1] If the migration occurs under exaggerated conditions the released compounds are termed extractables, whereas migrants that contaminate the actual DP under normal processing conditions, thus posing an increased risk for potential administration into patients, are referred to as leachables. Therefore, their contamination must be controlled in order to ensure adequate safety and quality of the final DP. The US Code of Federal Regulations Title 21 demands that manufacturing equipment[2] or containers and closures[3] "... shall not be reactive, additive, or absorptive so as to alter the safety, identity, strength, quality, or purity of the drug ... beyond the official or ... established requirements.". The attributes "reactive" and "additive" concern the phenomenon of leaching, potentially leading to alteration of safety, quality and purity.

The control of leachables from container closure systems is further specified in US[4] or European[5] guidance documents. The two USP chapters (<1663>[6] and <1664>[7]) give further guidance and suggestions on how to assess the leachables risk and how to perform extractables and leachables (EL) tests on container closure systems from the high risk category.

Abbreviations: ACN, Acetonitrile; APCI, Atmospheric pressure chemical ionization; API, Active pharmaceutical ingredient; BHT, Butylated hydroxytoluene; BPOG, BioPhorum Operations Group; DCM, Dichloromethane; DNPH, 2,4-dinitrophenylhydrazine; DP, Drug product; ESBO, Epoxidized soybean oil; HNE, 4-hydroxynonenal; MeOH, Methanol; m/z, mass to charge ratio; PA, Polyamide; PES, Polyethersulfone; PP, Polypropylene; PS, Polysorbate or Tween; PTFE, Polytetrafluoroethylene; PUFA, polyunsaturated fatty acid; PUR, Polyurethane; PVC, Polyvinylchloride; TIC, Total ion current; XIC, Extracted ion chromatogram.

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https://doi.org/10.1016/j.xphs.2021.05.014
0022-3549/

For manufacturing materials currently no Health Authority guidance documents are available, but harmonization of work practices is orchestrated within industrial working groups. For EL assessment of manufacturing materials for Biologics the BioPhorum working group (BPOG) has published a white paper outlining the principles of risk evaluation and testing strategies.[8] Notable is the importance of the risk parameter "distance along the production stream", which takes into account the possibility of the clearance of once introduced leachables during further purification process steps. The closer a material is located to the final DP, the higher is the risk that introduced leachables would be administered into the patient without further clearance. Extrapolating this logic beyond the manufacturing process, the materials with the highest risk according to the "distance along the production stream" should be administration materials, such as intravenous infusion bags, disposable syringes, administration catheters and in-line filters. Because clearance of leachables from those materials is not feasible under intended conditions, the migrating chemicals might come into direct contact with the patient. It should be mentioned that also under non-intended conditions, i.e., in the absence of dedicated clearance steps, clearance is possible due to non-specific adsorption of leachables to surfaces.[9]

Nonetheless, the topic EL on administration sets is not unequivocally covered in the regulatory landscape. As administration materials are classified as medical devices, EL testing is consequently performed according to the intended administration purpose, e.g., in case of IV bags with the contained solutions, such as aqueous 5% dextrose or 0.9% NaCl solution. However, when the infusion solution is mixed with a DP formulation or a DP is administered, the presence of the API or other formulation ingredients might cause a change in the leachables profile. Recently, a study on the extractables profile of disposable plastic syringes of clinical grade using different extraction solvents was published concluding that different solvents and solvent polarities could affect the extraction propensity regarding both amount and type of extractables.[10] Therefore, the test results obtained from the manufacturer certifying compliance cannot always be considered representative.

Besides the risk of administration of these contaminants into patients, their occurrence might also have direct impact on the stability and quality of a DP. For example, 2-mercaptobenzothiazole was observed to leach from saline-containing infusion bags, which unfavorably affected the stability of the recombinant therapeutic protein dulanermin.[11]

We have commenced a scientific study in order to test several widely used administration materials for leachable compounds. The main focus was on the administration of biopharmaceuticals. Biopharmaceutic DPs contain beside the API, i.e., the therapeutic protein, a combination of excipients, typically including non-ionic surfactants, such as polysorbate (PS) 20 or 80. PS20 and PS80 are commonly used to stabilize therapeutic proteins against interfacial stress and surface adsorption.[12-15] In addition to the protein stabilizing effect, they also act as solubilizers for non-polar compounds, which leads to an increased leachables propensity of the respective formulation compared to purely aqueous solutions.[16]

In order to test the materials generically, and to have the ability to apply the results to various administration scenarios, a relevant worst case approach regarding incubation conditions was pursued. The materials were subjected to a simulated in-use leachables study, with 0.1% (w/v) aqueous PS20 serving as the incubation solution, and with incubation times and temperatures chosen to mimic actual clinical practice. It should be noticed that the concentration span of PS in biopharmaceutical products typically varies between 0.001% (w/v) and 0.1% (w/v)[16-18] and that it may or may not be diluted with, e.g., the aqueous solution contained in potentially used IV bags. Therefore, using PS at the upper limit of commonly used concentration range can be considered as a representative and realistic worst-case DP surrogate solution.

The incubation solutions were quantitatively analyzed against analytical evaluation thresholds (AETs) that corresponded to relevant toxicological thresholds derived from the ICH M7 guideline.[19]

During the course of such a simulated in-use leachables study, the LC-UV-MS chromatograms of the extracts of three different IV administration sets showed a compound above the AET, which in contrast to other compounds detected above the AET could not be correlated to chemicals used during the manufacturing of plastics, such as plastic additives or related degradation products. The mass spectral data could not be correlated with other mass spectrometric methods applied in this study, such as GC-MS or HS-GC-MS, thus did not provide further information on the identity of the unknown compound. Consequently, the objective of the here presented study was to identify and quantify the detected leaching compound to enable its toxicological evaluation.

Materials and Methods

Chemicals, Reagents and Other Materials

HNE was purchased from Merck (Zug, Switzerland). 0.2 M 2,4-dinitrophenylhydrazine (DNPH) in phosphoric acid solution and polysorbate 80 (low peroxide) were purchased from Sigma Aldrich (Buchs, Switzerland). Polysorbate 20 (J.T. Baker grade) and polysorbate 80 (J.T. Baker grade) manufactured by Croda (Reinach, Switzerland) were purchased from Thermo Fisher Scientific Schweiz AG (Reinach, Switzerland). All glass syringe (50 mL, Fortuna Optima) and Hamilton syringes (1000 μL) were purchased from VWR (Dietikon, Switzerland). All other chemicals were of analytical and solvents were of chromatographic grade.

Methods

Simulated Administration Leachables Study

Three administration sets (A-C, n = 1), (see Table 1 and Figure S1) were in scope of the simulated administration leachables study. All components of the sets, except IV bag #1, were CE certified, and are routinely used in clinical practice.

Experimental Set-Up Administration Set A & B

The IV bags (100 mL, #1 and #6) containing 0.9% (w/v) NaCl were emptied and refilled completely with a drug product (DP) surrogate solution, i.e., 0.1% (w/v) polysorbate 20 (PS20), using a silicone oil-free, all-glass syringe. The two administration sets (A and B) were assembled including the individual components described in Table 1 and illustrated in Figure S1. The prepared administration sets were connected to a peristaltic pump and the simulated administration was executed at RT under ambient light. The resulting extracts were collected in glass bottles, closed and frozen in an upright position at -20°C prior to further analysis.

Experimental Set-Up Administration Set C

The disposable administration syringe (50 mL, #7) was completely filled with the same DP surrogate solution used for administration set A & B, i.e., 0.1% (w/v) PS20, and together with the other components assembled to administration set C (see Table 1 and Figure S1). The prepared administration set was connected to a peristaltic pump and the simulated administration was executed at RT under ambient light. The resulting extract was collected in a glass bottle, closed and frozen in an upright position at -20°C prior to further analysis.

Table 1
Overview on the three investigated administration sets. Individual administration components, assigned component numbers and the polymeric composition of the material that came into contact with the drug product surrogate formulation are shown.

Set	Administration component	Component number (#)[1)]	Polymeric composition of material
A	Saline IV bag (100 mL)	1	Polyvinylchloride (PVC)
	Administration line for IV bags	2	PVC
	In-line filter	3	Polyethersulfone (PES) membrane (neutral)
	3-way stopcock	4	Polyamide (PA)
	Catheter	5	Polyurethane (PUR)
B	Saline IV bag (100 mL)	6	Polypropylene (PP)
	Administration line for IV bags	2	PVC
	In-line filter	3	PES membrane (neutral)
	3-way stopcock	4	PA
	Catheter	5	PUR
C	Disposable syringe (50 mL)	7	PP
	Administration line for syringe	8	PVC
	In-line filter	9	PES membrane (positively charged)
	3-way stopcock	4	PA
	Catheter	5	PUR

1) *All components, except #1, were purchased from the same vendor and were CE certified.*

LC-UV-MS Analysis of Administration Set Extracts

For the analysis by LC-UV-MS, the three extracts of the simulated administration leachable study were thawed and subjected to liquid-liquid extraction. As a negative control 0.1% (w/v) aqueous PS20 solution was worked-up simultaneously. 10 g of each sample solution were weighed in a glass vial (20 mL). Afterwards 0.3 g of solid NaCl and 0.1 mL of a 10% (w/v) aqueous PS20 solution were added. The prepared samples were extracted three times by 1 mL dichloromethane (DCM) introduced with Hamilton syringes (1000 μL). For each extraction, the aqueous and organic phases were mixed using glass Pasteur pipettes and centrifuged for 10 min at 3000 G. Then the lower DCM phase was withdrawn using Hamilton syringes (1000 μL).

The three collected DCM volumes per sample were merged and the solvent was evaporated overnight. The samples were dissolved in 0.5 mL of a mixture of 95 volume parts of 10 mM ammonium formate and 5 volume parts of MeOH using a Hamilton syringe and analyzed.

Chromatographic separation and detection of the extracted and concentrated leachables was performed on an Orbitrap Fusion Lumos MS detector (Thermo Fisher, Basel, Switzerland), interfaced with UHPLC Vanquish liquid chromatography system including a diode-array detector (DAD) module (Thermo Fisher, Basel, Switzerland) using a Luna 3u C8(2) 100 A, 50 × 2.00 mm column (Phenomenex, Basel, Switzerland). The column temperature was set to 40°C. Injection volume was 25 μL. The flow was set to 0.4 mL/min. Eluent A was 10 mM ammonium formate in H_2O and B - MeOH. The following gradient was used: for 1 min 5% B, then in 7 min to 100% B, then for 4 min at 100% B. The total cycle time per injection was 12 min.

Mass analysis (atmospheric pressure chemical ionization (APCI) in positive mode) was applied in total ion current (TIC), with detection of m/z = 100 - 2000. In addition, UV (190 - 400 nm) was used for detection. The resulting chromatograms were analyzed at an absorption wavelength at 220 nm.

Confirmation and Quantification of HNE Leaching

To confirm the presence of HNE as a leachable of the three administration sets and to quantify the leaching amount, the obtained extracts of the simulated administration leachable study were thawed. HNE reference standards (2 - 50 ppb) were prepared in aqueous surfactant solution. The extracts and reference standards were worked-up and analyzed according to the HNE-DNPH assay that we had recently established.[20]

To an aliquot of the samples the same volume of the 0.05 M DNPH derivatization reagent (~pH 1, 0.2 M DNPH in phosphoric acid solution diluted by acetonitrile (ACN)) was added, and the mixture was incubated for 20 min at RT. One volume of 10 M NaOH was mixed with eight volumes of the derivatized samples leading to an exothermic neutralization reaction, which was controlled by storing the samples for 20 min at 2-8°C, and phase separation. The samples were centrifuged, and the resulting precipitate-free upper organic phases were transferred into HPLC vials and analyzed.

Chromatographic separation and detection of the formed HNE-DNPH hydrazones was performed on a QTRAP 6500 (Sciex, Baden, Switzerland) interfaced with 1290 Infinite UHPLC modules (Agilent, Basel, Switzerland) using a Luna 3 μm C8(2) 100 A, 50 × 2.00 mm column (Phenomenex, Basel, Switzerland) at a column temperature of 40°C. Injection volume was 20 μL. The flow was set to 0.25 mL/min. Eluent A) was 0.03% (v/v) acetic acid in H_2O and B) was ACN. The following gradient was used: in 8.5 min from 60% A to 35% A, then in 4 min to 0% A, then for 2.5 min at 0% A, then within 2 min up to 60% A, then for 3 min at 60% A. The total run time per injection was 22 min including 2 min DAD equilibration. Mass analysis (electrospray ionization (ESI) in negative mode) was applied using a multiple reaction monitoring (MRM) approach monitoring the HNE-DNPH hydrazone specific precursor/product ion transitions 335 > 167 and 335 > 163 using a collision energy of -24 V and a declustering potential of -50 V. Since similar results were obtained for the two transitions, only data obtained from the main transition, i.e., 335 > 167, showing a higher signal intensity, were reported in the course of this manuscript.

Screening Experiment Determining Individual HNE-Releasing Components

The 0.9% (w/v) NaCl containing IV bags were emptied with a silicone oil-free, all-glass syringe. An aliquot of each saline solution was analyzed without further preparation. To the emptied IV bags and other components of the three administration sets 1 mL of methanol (MeOH) was introduced by Hamilton syringes. Throughout the incubation time of ~10 min, the frequent contact of the solvent to all infusion-relevant surfaces of the components was ensured, e.g., by gently shaking of the components such as the IV bags and actively pushing the solvent through other components, e.g., the administration lines. Single extractions were performed. HNE reference standards (10 to 100 ppb) were prepared in MeOH.

Chromatographic separation and detection was performed on 1290 Infinite UHPLC modules including a DAD (Agilent, Basel, Switzerland) using a Luna 3u C8(2) 100 A, 50 × 2.00 mm column (Phenomenex, Basel, Switzerland) at a column temperature of 40°C. Injection volume was 25 μL. The flow was set to 0.4 mL/min. Eluent A) was 10 mM ammonium formate in H_2O and B) was MeOH. The following gradient was used: for 1 min 5% B, then in 7 min to 100% B, then for 4 min at 100% B. The total cycle time per injection was 12 min. UV (190 - 400 nm) was used for detection. The resulting chromatograms were analyzed at an absorption wavelength at 220 nm.

Confirmation of Suspected HNE-Releasing Components

To the three suspected HNE-releasing administration components, i.e., emptied IV bag (#1) and both administration lines (#2 and #8), 2 mL of 0.1% (w/v) PS20 were added and the contact of the solvent to all infusion-relevant surfaces of the components was ensured. HNE reference standards (30 and 100 ppb) were prepared. As a negative control, ACN was added in the same volume ratio to 0.1% (w/v) PS20 as present in the positive controls containing spiked HNE. The obtained extracts and controls were subjected to the DNPH-derivatization procedure and LC-MRM analysis as described above.

Results

A simulated in-use leachables study on various administration materials was conducted to quantify and identify potential leachables migrating during the infusion process from the administration materials into the drug product (DP) containing infusion solution. Within this study three administration sets were examined that consisted of components commonly used in clinical practice for the administration of biopharmaceutical DPs into patients. Table 1 provides a detailed overview of the administration sets (A-C), including information on the individual components, the assigned individual component numbers (#), and the polymeric composition of the materials in contact with the surrogate solution during the administration process, as obtained from the manufacturer. The simulation of the administration process was performed under real-use conditions using the assembled administration components and 0.1% (w/v) PS20 as a DP surrogate solution. The assembly of the three administration sets is schematically shown in Figure S1.

The obtained administration set leachables solutions were concentrated and analyzed by LC-UV-MS in high resolution (HR) mode to enable structure elucidation of potentially detected leachables (for details, see Materials and Methods). One of the compounds present above the AET in all three extracts, but not detectable in the negative control, revealed a UV spectrum with maximum absorbance at a wavelength of 220 nm. For illustration, the LC-UV-MS leachables profile of administration set A versus a simultaneously worked-up blank sample at the retention time window of the detected unknown leachable are shown in Fig. 1.

The corresponding HR-MS (see Fig. 1A3) of the detected UV peak at an absorbance wavelength of 220 nm (see Fig. 1A1), eluting at ~6.6 min, showed that the unknown compound had prominent signals at m/z = 139, 157 and 171 with proposed molecular formula of $C_9H_{15}O$, $C_9H_{17}O_2$ and $C_{10}H_{19}O_2$, respectively. The resulting extracted ion chromatograms (XIC) showed a good correlation, i.e., retention time shift of ~0.04 min due to the delayed MS detection, to the detected UV peak (see Fig. 1A2). In contrast, no UV and MS responses were detected in the worked up blank (see Fig. 1B1-3).

In retrospect, the mass spectral interpretation would not have been trivial, as will be shown further below. However, at the time point of this observation we were rewarded with the structural identification of the leachable compound based on a different study, where we identified 4-hydroxynonenal (HNE, $C_9H_{17}O_2$, molecular weight (MW) = 156 g/mol, Fig. 2) as an oxidative degradation product of PS80.[20]

Indeed, the obtained LC-UV-MS characteristics of the leachable compound, i.e., retention time, UV and HR-MS spectrum, matched with those of the authentic HNE reference.

The easily misleading detected MS signal at m/z = 171, corresponding to the sum formula $C_{10}H_{19}O_2$, was previously reported as an adduct formed by the reaction of the highly reactive α,β-unsaturated aldehyde with MeOH contained in the elution buffer, showing a specific fragment at m/z = 139 (formal loss of MeOH, MW = 32 g/mol), while the MS signal at m/z = 157 was assigned to the original chemical structure of HNE in positive ionization mode.[21, 22]

Because of the toxicity and reactivity of HNE (vide infra) and its detection at concentrations above the AET indicating a negative impact on patients' health, an additional experiment was performed to verify the unbelievable finding. The extracts of the administration sets (and HNE reference standards) were analyzed with an assay that

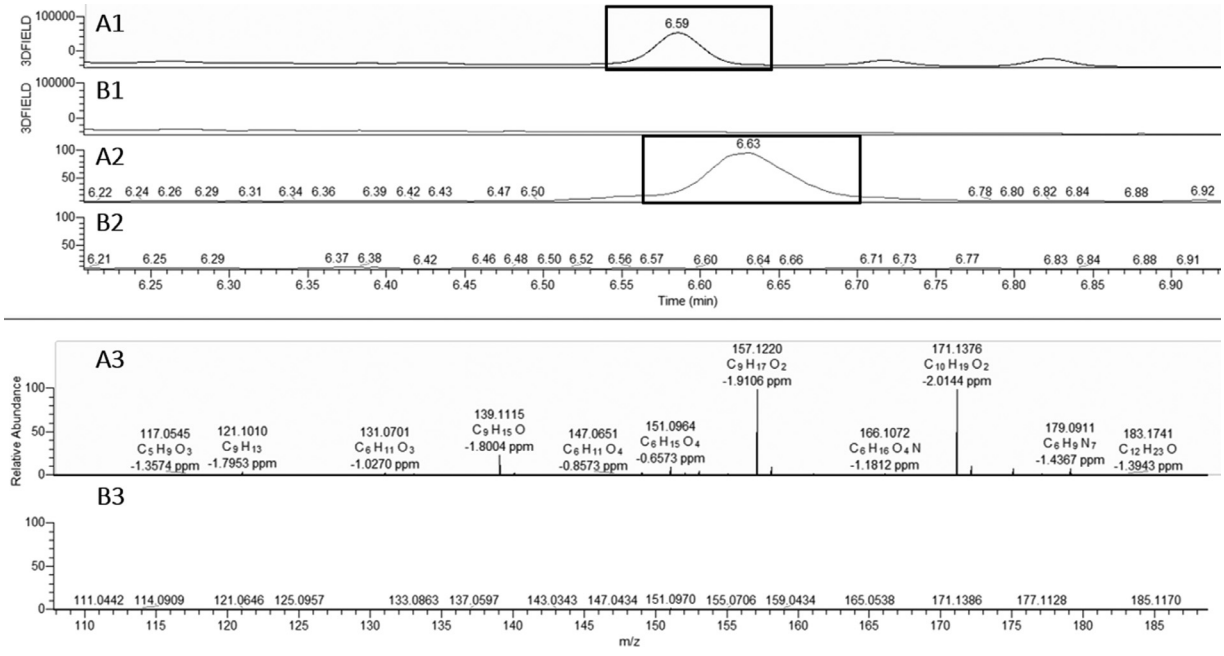

Figure 1. LC-UV-MS results of the leachable detected in the extracts of the administration sets. Results of administration set A (A) and a blank (B), i.e., worked up 0.1% (w/v) PS20, are shown.[1] UV chromatogram at an absorbance wavelength at 220 nm showing a peak, i.e. a leachable (black framed), eluting after 6.59 min in the leachables solution.[2] Extracted ion chromatogram (XIC) at m/z = 157 showing a peak correlating well with the detected UV peak in the leachables solution (with the typical ~0.04 min delay between UV and MS detection).[3] The corresponding high-resolution mass spectrum at retention time ~6.63 min showing prominent signals at m/z = 139, 157 and 171 (Δ ~2 ppm) with proposed molecular formula of $C_9H_{15}O$, $C_9H_{17}O_2$ and $C_{10}H_{19}O_2$, respectively.

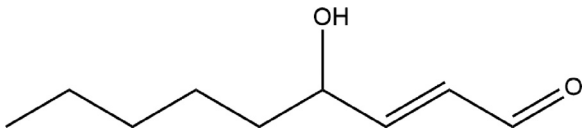

Figure 2. Chemical structure. 4-hydroxynonenal (HNE).

we had recently developed and qualified to specifically allow the detection and precise quantification of HNE in a complex matrix, such as PS.[20] In brief, the assay is based on the derivatization of the aldehydic moiety by 2,4-dinitrophenylhydrazine (DNPH) forming a hydrazone, followed by a quenching step simultaneously leading to phase separation. The resulting upper organic phase containing the stabilized hydrazone was analyzed by LC-MS using a multiple-reaction-monitoring (MRM) approach monitoring its specific precursor > product ion transition at m/z 335 > 167.

The overlay of the obtained MRM signals of the formed HNE-DNPH hydrazones showed that HNE was present in the extracts of all investigated administration sets, although at different concentration levels (see Fig. 3).

The total amount of leaching HNE per administration set was calculated by interpolating the average MRM area of the injected triplicates into the obtained linear calibration curve ($R^2 > 0.99$), followed by multiplication of the resulting concentration (ppb) with the volume of the incubation solution, i.e., for administration set A and B by 100 mL and for set C by 50 mL. The total amount of HNE leaching from administration set B and C was above the applied AET (15 ppb in 100 mL incubation solution, and 30 ppb in 50 mL, respectively), corresponding to the safety concern threshold (SCT) of 1.5 μg, i.e., the leachable amount was at 4.6 μg and 1.9 μg, respectively, both with relative standard deviations < 2% (see Fig. 3B). Also administration set A leached HNE, but below the reporting limit of the calibration curve.

To determine the primary HNE releasing components, the total of nine components of the administration sets (see Table 1) were extracted individually with an organic solvent compatible with the analytical method, i.e., MeOH. Single extractions were performed using Hamilton syringes to introduce 1 mL of the organic solvent to each component. The relatively low incubation volume was used to mitigate dilution effects of potentially leaching HNE. To address differences in the surface areas, frequent contact of the solvent with all relevant surfaces of the individual components was ensured throughout the incubation period of ~10 min, e.g., by gently shaking the refilled IV bags or actively pushing the MeOH through the other components such as the administration lines. In addition, aliquots of the originally contained and removed saline solutions of the two IV bags were analyzed for the presence and quantity of HNE. Quantification was performed against HNE reference standards in MeOH. The samples and reference standards were analyzed by LC-UV at a wavelength of 220 nm, i.e., the absorbance maximum of HNE (see Figure S2). Table 2 summarizes the results.

Only three of the nine components showed the presence of HNE, namely one of the two IV bags (#1) and both administration lines (#2 and #8). Additionally, HNE was detected and quantified from the infusion solution originally contained in bag #1 (i.e., #1*) by interpolating the average UV area of the injected triplicates into the linear equation of the calibration curve ($R^2 > 0.99$), and multiplying the resulting concentration (ppb) by the volume of the removed saline solution, i.e., 100 mL. The quantified amount of HNE contained in the 100 mL saline solution was at ~3 μg (n = 3, SD ± 1.4%), which is above the toxicological threshold of ICH M7 guideline at 1.5 μg.

The leaching amount of all other HNE positive components was not quantitatively evaluated, because complete and comparable removal of the incubation solutions, i.e., the MeOH extracts, was not possible.

For verification of the suspected materials and to evaluate the HNE leaching propensity into DP formulations the three HNE positive administration components were extracted with the previously used worst case DP surrogate solution, i.e., 0.1% (w/v) PS20. The obtained extracts and reference standards were analyzed by the HNE-DNPH assay (see above). As expected, the determined MRM chromatograms confirmed the three components as primary HNE releasing source as well as the quick leaching propensity of HNE into drug product formulations when contacting these components (see Figure S3). Interestingly, according to the information provided by the manufacturers, all HNE releasing materials were made from PVC (#1, #2 and #8), while all other components made from different polymers did not release HNE (see Table 1).

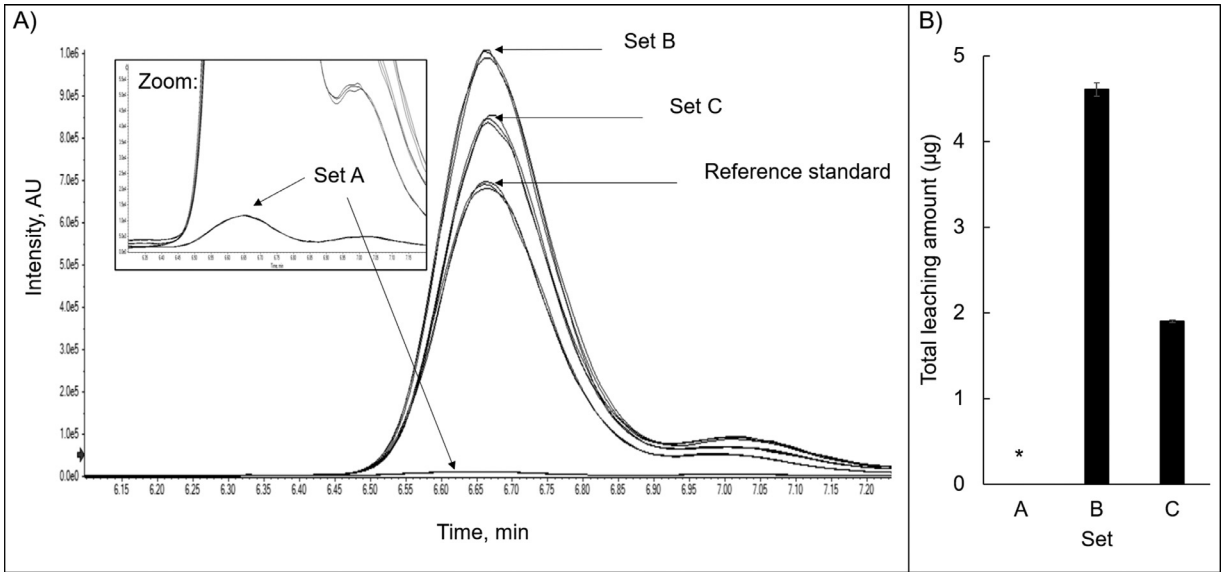

Figure 3. Confirmation of HNE as a leachable of the administration sets. A) A LC-MS overlay of HNE-DNPH hydrazone MRM signals for the transition 335 > 167 of the worked-up extracts of administration set A, B and C, and a reference standard containing 30 ppb HNE is shown (triplicate injections). B) The calculated total leaching amount of HNE per administration volume of the respective administration set is shown (n=3 injections, SD < 2%). Asterisk for set A: calculated concentration / amount of HNE was below the reporting limit.

Table 2
Results of primary HNE-releasing component screening study. Presence or absence of the HNE UV signal at an absorbance wavelength of 220 nm in the MeOH extracts of individual administration components (#1-9) and withdrawn saline solutions from the two IV bags (#1* and #6*) is shown (n = 3 injections).

Administration component	Component number (#)[1)]	Presence (P) / absence (A) of HNE [2)]
Saline IV bag (100 mL)	1	P
Saline IV bag (100 mL)	1*	P (~3 μg in 100 mL, n = 3, SD ± 1.4%)
Administration line for IV bags	2	P
In-line filter	3	A
3-way stopcock	4	A
Catheter	5	A
Saline IV bag (100 mL)	6	A
Saline IV bag (100 mL)	6*	A
Disposable syringe (50 mL)	7	A
Administration line for syringe	8	P
In-line filter	9	A
3-way stopcock	4	A
Catheter	5	A

1) *Asterisk: saline solution originally contained in the IV bags.*
2) Only the *HNE content of the saline solution of infusion bag (#1*) was quantifiable, because accurate removal of the incubation solutions from the other HNE positive components was not possible.*

Discussion

HNE was previously reported to be a main oxidation product of n-6 polyunsaturated fatty acids (PUFA), such as linoleic acid.[23-25] HNE is involved in a great number of pathologies, such as metabolic diseases, neurodegenerative diseases and cancers.[23,26-28] Moreover it was detected in various food products,[25] such as vegetable oils rich in n-6 PUFA, e.g., soybean oil,[29] oil-based food, e.g., fried potatoes,[30] and dry nuts, e.g., peanuts.[31] In correlation with the here reported leaching propensity of HNE from administration materials, its recent detection as an oxidative degradation product of PS80 could in particular be of greater relevance to patients' health.[20] With regard to the leaching propensity of DP formulations, the two common micelle-forming surfactants likely exhibit comparable properties. Therefore, PS80-containing DPs may pose an increased risk to patients, as these products could constitute an additional source of HNE due to the surfactant's known susceptibility to oxidation.[32,33]

As a 4-hydroxy-alkenal, the non-2-ene part of the molecule consists of an oxo group at the 1-position and a hydroxy group at the 4-position (Fig. 2), making it a highly electrophilic chemical. A resulting special feature of α,β-unsaturated aldehyde is its ability to simultaneously participate in Michael reactions and Schiff base formations with nucleophilic counterparts such as amino and thiol groups, e.g., present in nucleic acids[23,26,27] and amino acid residues of proteins.[23,25,34] Due to this bi-reactivity, HNE can act as a crosslinking agent of susceptible molecules in addition to covalent adduct formations.[27,35,36] This could also relate to therapeutic proteins and other APIs containing vulnerable moieties.

In addition, HNE was found to have potential to chemically alter DNA.[26,27,37,38] One of the first studies to reliably demonstrate the genotoxicity of HNE has already been published in 1993.[37] Within that study, the genotoxic effect of HNE was investigated in rat hepatocytes and, e.g., indicated by sister chromatid exchange and chromosomal aberrations. Furthermore, according to Feng et al.[26], HNE may play an essential role in carcinogenesis as it is able to interact with DNA to form 6-(1-hydroxyhexanyl)-8-hydroxy-1,N(2)-propano-2′-deoxyguanosine (4-HNE-dG) adducts. The study showed that 4-HNE-dG adducts were mutagenic in human cells. Additional examples indicating the genotoxic potential of HNE can be found in the comprehensive review article of Eckl and Bresgen.[38] Therefore, the leaching HNE has to be considered genotoxic. ICH M7 defines a regulatory limit of 1.5 μg/day for directly acting agents on DNA. Referring to this threshold of toxicological concern (TTC) the acceptable daily intake of 1.5 μg of a mutagenic compound is considered to be associated with a negligible cancer risk (1:100′000). Because the determined leaching concentrations of potentially genotoxic HNE were above the relevant toxicological thresholds derived from the ICH M7[19], the health risk of patients could be directly affected.

It should be mentioned that the applied extraction conditions of the in-use leachables study were chosen in order to simulate a clinical worst case scenario, i.e., the IV bags were completely filled with DP surrogate solution instead of diluting it with the contained saline solution and the first few volumes of the infusion solution were not discarded as a preflush. Nevertheless, the impact of the worst case scenario conditions on the determined total leaching amount is probably negligible, as HNE also leached in concentration above the applied AET - and thus above the TTC - into the pure aqueous saline solution of IV bag (#1*).

In our recently published study, we identified HNE as an oxidative degradation product of PS80 using the above described HNE-DNPH assay.[20] Thus, the previously obtained results could suggest that the detection of HNE in the leachables solutions was comparatively due to the degradation of PS20 species contained in the DP surrogate formulation and not due to the its leaching from the administration set materials. However, the used multi-compendial PS20 can be excluded as HNE source, which is related to the negligible amount of n6-polyunsaturated fatty acids, i.e., linoleic acid, being known precursors of HNE. As specified by the pharmacopeias (EP, USP, JP) linoleic acid might account for up to 18% in PS80 products while only up to 3% are acceptable in PS20 products.

Furthermore, the confirmed leaching of HNE into surfactant-free matrices, such as the methanolic extracts as well as the saline solution contained in the HNE-positive IV bag, provides further evidence that it is a genuine leachable. Since only single sets were in scope of the present study evaluating their potentially occurring leachables profile during clinical use, further studies should investigate batch to batch variabilities as well as other administration components consisting of related materials. In this course, another interesting aspect could be to examine whether a correlation can be established between the HNE detection and the sterilization process used for the individual components.

Nevertheless, the determined HNE leaching from administration set components can be considered significant, as three different components from different vendors were independently confirmed as primary HNE-releasing source.

To summarize, leaching HNE from administration set components above the commonly applied toxicological thresholds into aqueous DP formulations is critical from several points of view and should therefore be controlled and reduced to a minimum.

Conclusion

To the extent of our knowledge, a novel and toxicologically concerning leachable from clinically relevant administration materials was identified within the here presented study, namely the highly reactive and potentially genotoxic α,β-unsaturated aldehyde HNE. The total leaching amount determined in the DP surrogate solutions as well as in the saline solution contained in an infusion bag exceeded the critical toxicological threshold (TTC) deduced from ICH M7. Hence, its leaching propensity implies an increased safety risk to patients. Due to the discussed genotoxicity and reactivity of the chemical, it should be in the interest of the manufacturers to conduct further root cause investigations to enable the implementation of mitigation strategies to ensure patients safety beside avoiding the

use of such components for DP administration purposes. As the primary HNE releasing components were all made of PVC the root cause of leaching HNE might be related to the plastic composition or manufacturing. In this respect, we recommend to especially consider one group of plastic additives, namely epoxidized vegetable oils, such as epoxidized soybean oil (ESBO).[39] The PVC plasticizer consists to a large extent of n-6 PUFAs, such as linoleic acid,[39] which are known to form HNE during oxidative degradation.[23-25] Furthermore, HNE was shown to be formed as a degradation product of the related non-epoxidized soybean oil.[25, 29] Nevertheless, the assumption made that such n-6 PUFA containing plasticizers are the precursor of the leaching HNE is speculative and requires further confirmatory experiments.

To conclude, the leaching of HNE from PVC administration materials should be properly understood, monitored, e.g., using our recently developed sensitive HNE-DNPH assay, and reduced to a minimum in order to limit HNE leaching into (drug) product solution and consequently administration into patients.

Author Agreement

All authors have approved the manuscript and agree with its submission to Journal of Pharmaceutical Sciences.

Conflict of Interest

The authors declare that there is no conflict of interest.

Statement on Funding

This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

Statement on Research Data

Research data used in preparation of the manuscript is available, and can be requested from the corresponding author.

Declaration of Competing Interests

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgments

The authors would like to thank Ahmed Besheer for the critical review of the manuscript, Selene Araya for toxicological evaluation and the Forensic Chemistry group of Lonza Drug Product Services for scientific discussions and general support.

Supplementary Materials

Supplementary material associated with this article can be found in the online version at https://doi.org/10.1016/j.xphs.2021.05.014.

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