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Isolation, structural determination, synthesis and quantitative determination of impurities in Intron-A, leached from a silicone tubing

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ABSTRACT

Investigation of unexpected levels of impurities in Intron product has revealed the presence of low levels of impurities leached from the silicone tubing (Rehau RAU-SIK) on the Bosch filling line. In order to investigate the effect of these compounds (**1a**, **1b** and **2**) on humans, they were isolated identified and synthesized. They were extracted from the tubing by stirring in Intron placebo at room temperature for 72 h and were enriched on a reverse phase CHP-20P column, eluting with gradient aqueous ACN and were separated by HPLC. Structural elucidation of **1a**, **1b** and **2** by MS and NMR studies demonstrated them to be halogenated biphenyl carboxylic acids. The structures were confirmed by independent synthesis. Levels of extractable impurities in first filled vials of actual production are estimated to be in the range of 0.01–0.55 μg/vial for each leached impurity. Potential toxicity of these extractables does not represent a risk for patients under the conditions of clinical use.

1. Introduction

Intron-A is a lyophilized interferon-α2b powder for injection approved for several indications including hepatitis-C and melanomas. HPLC analysis of vials of pre-lyophilized Intron solution dispensed immediately from the filling line after extended hold time revealed the presence of two new peaks as shown in Fig. 1. Further investigation revealed that these are leached impurities from the flexible silicone tubing (Rehau RAU-SIK) in the fill-line and that Peak 1 consists of two leachables. Quantitation of Peak 1 and Peak 2 in actual vials filled after fill-line stoppages showed that these leachables can be present at levels of about 0.01–0.55 μg/vial.

In order to identify these impurities, a length of silicone tubing was extracted with buffer, the extract was concentrated and the impurities were isolated by HPLC. Three impurities were purified and characterized by MS and NMR. The structures of these impurities revealed that they are formed during the polymerization process used to make the raw material for the tubes and are extracted by the buffered Intron-A solution. Normally the levels of these compounds in each vial of Intron-A are extremely small and undetectable. When the fill-line is stopped for an extended amount of time for any reason, these compounds accumulate in the static solution in the tubing and are dispensed into vials when the line resumes. Analysis of the vials filled immediately after the restart would show much higher levels of these impurities. The levels of these impurities then return to the normal low level when continuous flow resumes. The longer the stoppage, the higher are the levels of these accumulated impurities. Depending on the standard operating procedure of the manufacturing process and the method of QC testing, these abnormal levels may not be detected. Since this type of silicone tubing is used in fill-lines in many manufacturing processes and in medical equipment such as dialysis machines, these finding may be of interest to the scientific and medical communities. Preliminary toxicology studies indicate these three compounds are not toxic.

2. Experimental

2.1. Materials and methods

Rehau RAU-SIK silicone tubing (part no. 800770; 4 mm ID, 10 mm OD) was obtained from Rehau Ltd., Hill Court, Walford,

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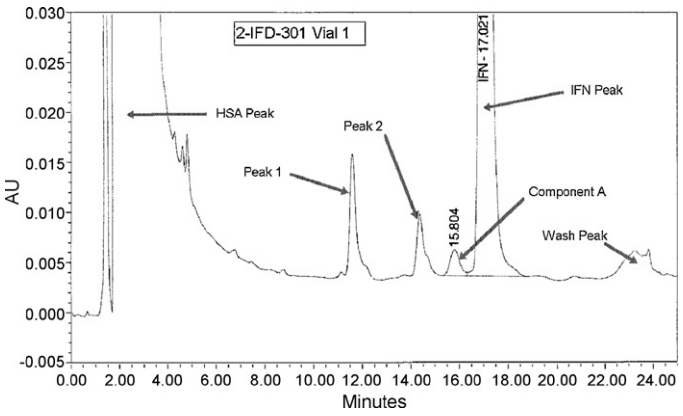


Fig. 1. A chromatogram of a first vial filled of Interferon-α2b showing the presence of the two additional peaks (Peak 1 and Peak 2).

UK. In the manufacturing process the Rehau RAU-SIK silicone tubing undergoes a treatment process before being used on the filling line (denoted as 'treated tubing'). This treatment involves 5× wet autoclave cycles followed by sterilization. DMSO-d6, bis(2,4-dichlorobenzoyl) peroxide and 2,4-dichlorobenzoic acid were obtained from ACROS Chemical Inc., acetonitrile (HPLC grade), carbon tetrachloride and all other chemicals were purchased from Fisher Scientific (Fair Lawn, NJ). All solutions were prepared in doubly deionized water and filtered through a 0.45-μm membrane.

2.1.1. High-performance liquid chromatography (HPLC)

The HPLC analysis system consisted of a Waters (Milford Mass.) system consisting of 600E pumps, 996 diode array detector monitoring at 254 nm and using Millennium 32 software. The HPLC analytical column Develosil RPAQ C30 column (Nomura Chemicals) and semi-preparative Luna C18 (Phenomenex, 5 μm, 10 mm × 250 mm) and preparative Luna C18 (Phenomenex, 21 mm × 250 mm) columns were obtained from Phenomenex corporation, CA and Vydac C18, 4.6 mm × 150 mm, 5 μm column was made by Vydac Inc.

2.1.2. Mass spectrometry

LC-ESI/MS (−ve) experiments were performed on a Micromass Quattro LC-triple quadrupole mass spectrometer (Manchester UK) with a Waters Alliance 2690 LC system (Milford, MA). Similar chromatographic conditions were used as described in the HPLC section. The mobile phase for the analysis of the three peaks contained 40% acetonitrile and 60% water. The flow rate was set at 1 mL/min. A 19:1 post-column split fed the larger volume to the UV detector set at 254 nm and the smaller volume in to the mass spectrometer. An electrospray (ESI) was used with nitrogen as the disolvation gas. The ESI voltage supplied to a stainless-steel needle was 3750 V and the cone voltage was set at 30 V. The temperature of the disolvation chamber was set at 350 °C and the source block was set at 100 °C. The first quadrupole mass analyzer was used for the LC–MS experiments. In order to optimize the fragmentation of the selected parent ions for MS/MS experiments, the collision (Ar) gas cell pressure was adjusted to 1.4×10^{-3} Torr. The selected parent ions from the first quadrupole mass analyzer were collided with Ar in the second quadrupole with collision energy of 20 V and the resulting product ions were scanned by the third quadrupole mass analyzer. Mass spectra were obtained by liquid chromatography (LC)-negative ion electrospray ionization (ESI) and electron ionization (EI)-mass spectrometry (MS) techniques. GC-high resolution (HR)–EI-MS experiments were performed on a JEOL GCmate mass spectrometer equipped with HP 6890 series GC. Ionization energy was set at 70 eV. The accelerating voltage was at 2500 V and the scan mode was in magnet. Leachable impurity **2** was analyzed as trimethylsilyl (TMS) and methyl ester derivatives by GC–EI-MS.

2.1.3. Nuclear magnetic resonance

Proton and Carbon NMR were performed on a GE 500 MHz instrument. Spectra were obtained in DMSO-d6 at 25 °C in a 3-mm probe. The isolated samples were about 0.5 mg and were dissolved in 100 μL of solvent and transferred to a 3-mm Shigemi NMR tube (Shigemi Inc., Allison Park PA). For the synthetic samples, about 5 mg of the sample were dissolved in 200 μL of solvent and transferred to a regular 3 mm NMR tube. One-dimensional (1D) proton NMR spectra were recorded into 32,000 data points with a spectral width of 9500 Hz and acquisition time of 1.7 s. A total of 2000 transients were collected in approximately 1.5 h for each 1D proton spectrum. Two-dimensional proton–proton TOCSY spectra were acquired using 96 scans per increment and the 256 hypercomplex data points in t_1 for a total collection time of 21 h. The values for the spectral width in both F1 and F2 dimensions were set to be 11,000 Hz, and the spin-lock time was 30 ms. For the HMBC spectra, the delay was set for a long-range coupling constant of 7 Hz, which will usually yield correlations for proton–carbon nuclei that are two or three bonds apart.

2.1.4. Laboratory studies to determine the origin of impurities & factors impacting impurity levels

To determine the origin of these impurities, a number of laboratory extraction studies were performed probing the contact of reconstituted Intron with various materials. All samples were analyzed by the QC RP-HPLC test method for Intron (Vydac C18 column, 4.6 mm × 150 mm). Using the QC HPLC method, the leachables eluted as two peaks (Fig. 1). More detailed extraction studies were also performed involving 22 °C incubation of Intron, Intron placebo [with and without HSA (human serum albumin)] and new treated (5× wet autoclave cycles) and untreated tubing from the vendor. Samples from the tubing were tested by HPLC at time 0, 2, 6, 24 and 48 h. Since the RAU-SIK silicone tubing is used to fill multiple batches of Intron, a laboratory extraction study under similar experimental conditions as above using a laboratory prepared Intron batch was also performed on tubing that had previously been used in production. In addition to the above studies using peroxide-cured Rehau technical grade RAU-SIK tubing, incubation at 22 °C was performed with new treated Rehau medical grade peroxide-cured RAUMEDIC-SIK silicone tubing using Intron placebo. Medical grade RAUMEDIC-SIK tubing is used in infusion pumps, dialysis machines and ECC (exocorporal circulation).

2.1.5. Extraction of Rehau Rau-SIK silicone tubing for quantitative isolation of leachables

Two 60 cm lengths of previously treated Rehau Rau-SIK silicone tubing (part no. 800770, 4 mm ID, 10 mm OD) were cut to 0.5–1.0 cm lengths and stirred with 1 L of buffer (2.27 g of NaH_2PO_4, 0.55 g Na_2HPO_4, 20 g of glycine in 1 L of water) at room temperature for 72 h. The solution was filtered and loaded on a reverse phase CHP-20 (Mitsubishi Chemicals) column (2.5 cm × 15 cm). The column was washed with 1 L of water to remove the buffer and then eluted with 1 L of acetonitrile. Extractable impurities **1a**, **1b** and **2** (5.5 mg) were obtained as a mixture after the solvent was removed. The individual peaks were separated by HPLC on a Develosil C30 preparative column (Nomura Chemicals, 10 mm × 250 mm), using 0.05% TFA (trifluoroacetic acid):ACN 40:60 solvent mixture. The individual peaks were collected separately and the samples were dried in a vacuum oven overnight at room temperature to yield 643.3, 683.3 and 663.3 μg of **1a**, **1b** and **2**.

Table 1
Evaluation of extractable Peak #1 in Rehau RAU-SIK tubing (P/N 800770).

Time (h)	Treated tubing placebo minus HSA (μg/mL) (TT placebo-HSA)	Treated tubing placebo (μg/mL) (TT placebo)	Treated tubing 2-IFNA-301 (μg/mL) (TT 2-IFNA-301)	Treated tubing WFI (μg/mL) (TT WFI)	Untreated tubing placebo minus HSA (μg/mL) (UnTT placebo-HSA)
0	0	0	0.00	0	0
2	0.88	1.24	0.75	0.42	1.85
6	1.28	1.69	1.22	0.42	3.02
24	2.59	2.86	2.60	0.36	6.68
48	3.85	4.07	3.97	0.34	10.06

Response factor calculated from purified **2** was applied; TT = treated tubing; UnTT = untreated tubing.

Table 2
Evaluation of extractable Peak #2 in Rehau RAU-SIK tubing (P/N 800770).

Time (h)	Treated tubing placebo minus HSA (μg/mL) (TT placebo-HSA)	Treated tubing placebo (μg/mL) (TT placebo)	Treated tubing 2-IFNA-301 (μg/mL) (TT 2-IFNA-301)	Treated tubing WFI (μg/mL) (TT WFI)	Untreated tubing placebo minus HSA (μg/mL) (UnTT placebo-HSA)
0	0	0	0	0	0
2	0.75	1.24	0.70	0.09	1.05
6	1.08	1.64	1.13	0.08	1.62
24	2.10	2.64	2.31	0.05	3.33
48	3.01	3.68	3.44	0.05	4.72

Response factor calculated from purified **2** was applied; TT = treated tubing; UnTT = untreated tubing.

2.1.6. Synthesis of *1a*, *1b* and *2*

Compounds **1a**, **1b** and **2** were prepared by heating a solution of the peroxide **3** and 2,4-dichlorobenzoic acid **6** in carbon tetrachloride. A solution of **3** (5 g, 13.2 mmol) and **6** (12.5 g, 65.6 mmol) in CCl_4 (100 mL) under an N_2 atmosphere was heated to reflux for 1 h and 75 °C for 41 h. The solvent was removed in vacuo and the products were purified by the procedure described below.

2.1.7. Purification of *1a*, *1b* and *2* from the synthetic mixture

The reaction products were dissolved in an acetone and methanol mixture and added to 20 mL of CHP-20 (Mitsubishi Chemicals). The mixture was dried and added as a plug on the top of a CHP-20 column, previously (5/8 in. × 25 in.) dry packed and equilibrated with a mixture of 0.05% TFA and ACN (80:20). The column was eluted with a stepwise gradient beginning with 0.05% TFA:ACN 80:20 to a final concentration of 0.05% TFA:ACN 40:60 and held at this concentration. The fractions enriched with **1a**, **1b** and **2** were combined separately and the solvent removed to yield 265.9 and 72 mg of fractions 1 and 2, respectively. Fraction 1 (265.9 mg) contained **1a** as the major component and **1b** and **2** in minor amounts. Fraction 2 (72 mg) contained mainly compound **2** and minor amounts of **1a** and **1b**. Fraction 1 (90 mg) was separated on a Phenomenex Luna C18, 5 μm, preparative HPLC column, 21 mm × 250 mm, eluting with a mixture of 0.05% TFA and ACN (40:60) gave 36.9, 7.1 and 15.0 mg of **1a**, **1b** and **2**, respectively. Fraction 2 (72 mg) was also separated on the same column using identical conditions yielded 5.8, 3.8 and 45.1 mg of **1a**, **1b** and **2**, respectively.

3. Results and discussion

3.1. Laboratory investigation of the origin of the impurities

The studies performed probing the contact of reconstituted Intron with various materials, revealed that the additional peaks come from the Rehau RAU-SIK silicone tubing used on the filling line.

Spiking experiments and A_{214}/A_{280} peak area ratio determination confirmed that the peaks extracted from Rehau RAU-SIK silicone tubing, in laboratory experiments are identical to the peaks observed in vials filled in production.

Table 3
Evaluation of extractable Peaks in used/aged RAU-SIK Tubing (P/N 800770).

Length	Incubation time (min)	Peak 1 (μg/mL)	Peak 2 (μg/mL)
1	15	0.08	0.03
2	30	0.11	0.04
3	45	N.D.	N.D.
4	60	N.D.	N.D.
5	120	N.D.	N.D.
6	360	N.D.	N.D.
7	4320	N.D.	N.D.

Response factor calculated from purified **2** was applied. For this study, a laboratory prepared 3 MIU/mL Intron powder for injection solution was incubated in separate lengths of used/aged tubing at 22 °C.

The evaluation of Peak 1 under different condition with the time is shown in Table 1 and for Peak 2 is shown in Table 2.

The results from the detailed extraction studies which included (1) time course incubation studies over 48 h at 22 °C of Intron (reconstituted vials from Intron batch 2-IFNA-301) and Intron placebo (with and without HSA) in new treated and untreated Rehau RAU-SIK silicone tubing (Tables 1 and 2), (2) incubation at 22 °C of a laboratory prepared Intron batch in Rehau RAU-SIK silicone tubing that had previously been used in production (Table 3), and (3) incubation at 22 °C of a laboratory prepared Intron placebo in new treated Rehau RAU-SIK silicone (technical grade) tubing and new treated Rehau medical grade RAUMEDIC-SIK silicone tubing (Table 4) used in infusion pumps, dialysis machines and ECC (exocorporal circulation) with Intron placebo established the following conclusions:

(1) The extraction process is not linear, with the highest rates being observed over the first 2 h.

Table 4
Comparison of extraction from RAUMEDIC-SIK treated tubing (P/N 819502) with extraction from Rehau RAU-SIK treated tubing (P/N 800770).

	Time (h)	Peak 1 (μg/mL)	Peak 2 (μg/mL)
Sample			
RAUMEDIC-SIK tubing	22	2.78	3.07
RAU-SIK tubing	24	2.86	2.64

Response factor calculated from purified **2** was applied. For this study, a laboratory prepared placebo solution was used.

(2) Higher levels of extractables are observed in untreated tubing vs. treated tubing.
(3) Studies on RAU-SIK tubing previously used in production (i.e. used tubing) showed lower or non-detectable levels of extractables than fresh treated or untreated tubing.
(4) The same level of extractables is observed in the both the medical grade Rehau RAUMEDIC-SIK tubing and in the technical grade Rehau RAU-SIK tubing.
(5) The presence of protein appeared to facilitate extraction of the leachables (placebo minus HSA is protein free).

3.2. Estimate of extractable concentration

When extractable impurities were detected in first vials filled of Intron by the QC HPLC assay method, they generally eluted as two peaks between the HSA peak and the interferon peak. These two additional peaks are denoted as Peak 1 and Peak 2. Peak 1 is composed of two extractable compounds **1a** and **1b**. The levels reported for Peak 1 represent the combined levels of **1a** and **1b**. For some batches a detectable level of Peak 1 was observed, while Peak 2 was not detected (ND). This was due to the fact that Peak 2 could co-elute with the component A peak (monosulphoxide variant of the active, Interferon-α2b) in the QC HPLC assay chromatographic method. In order to provide a "worst-case" estimate of the extractable impurities concentration under manufacturing conditions, a maximum contact time of 6 h was used. This is the maximum product contact time in the tubing under a filling line hold. For any filling hold longer than 6 h, the filling line is flushed prior to restart in order to remove any material that has been in the line during the holding period. The calculations to estimate a "worst-case" level of the extractable impurities utilized the highest level of any extractable found in the testing results from actual manufacturing samples, the corresponding time of the filling line hold, and the time-extraction profile results obtained from the laboratory simulation (Figs. 2 and 3). The maximum level of Peak 1 found in manufactured vials was 0.55 μg/vial. The start up hold time for this batch was 39 min. Using the time-extraction profile (Fig. 2), and the ratio of material extracted at 6 h vs. 39 min, an estimate of approximately 2.3 μg/vial at 6 h results. This result of 2.3 μg/vial for Peak 1 represents "worst-case" estimate of the sum of **1a** plus **1b**. The maximum level of Peak 2 found in actual manufactured vials was 0.33 μg/vial. The start up hold time for this batch was 2 h and 36 min. Utilizing above calculation method with the time-extraction profile (Fig. 3), results in 0.4 μg/vial at 6 h. Thus, a "worst-case" level of 0.4 μg/vial for Peak 2 was estimated.

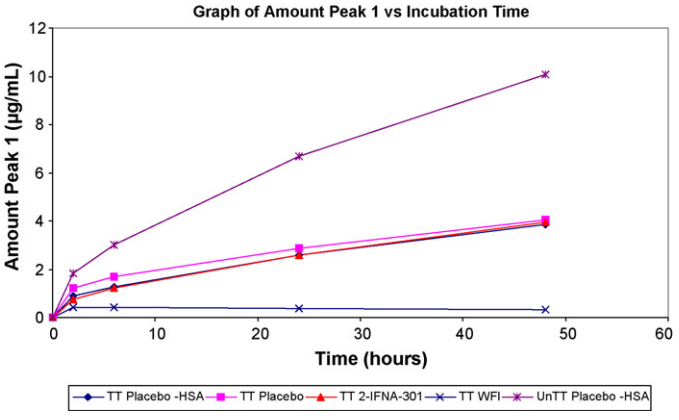


Fig. 2. Time-extraction profile for Peak 1.

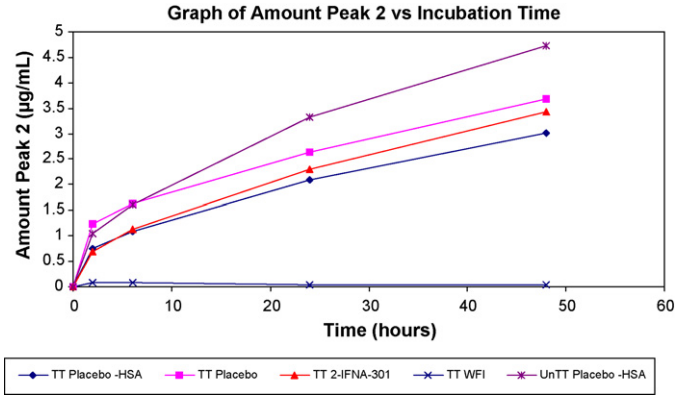


Fig. 3. Time-extraction profile for Peak 2.

3.3. Mass spectral analysis of 1a, 1b and 2 from extraction of Rehau Rau-SIK silicone tubing

The three impurities, **1a**, **1b** and **2** were analyzed by liquid chromatography (LC)-negative ion electrospray ionization (ESI) and electron ionization (EI)-mass spectrometry (MS) techniques. LC–ESI-MS of each sample produced an intense peak in the total ion chromatogram. The mass spectrum of each of these peaks yielded $(M-H)^-$ signal at *m*/*z* 333 with an abundant fragment ion at *m*/*z* 289. The isotope patterns of both the molecular ion and the fragment ion represent the presence of four chlorine atoms in the compounds. This data established the molecular weight of each impurity as 334 Da and that all the three compounds are isomers. The fragment ion at *m*/*z* 289 represented the loss of 44 Da, which indicated the presence of a carboxyl (−COOH) function in the molecule. Compound **1a** was also analyzed as the methyl ester by GC–EI-MS which displayed a molecular ion with a four chlorine isotope pattern at *m*/*z* 347.9 (348). HR–EI-MS of the methyl derivative provided a measured molecular mass of 347.9322 Da and this measured value gave the best possible elemental composition of $C_{14}H_8O_2Cl_4$ (14.11 ppm). Thus, the elemental composition of compound **1a** was suggested to be $C_{13}H_6O_2Cl_4$.

Extractable impurity component **2** was analyzed as trimethylsilyl and methyl ester derivatives by gas chromatography–positive ion EI-MS. The TMS derivative produced the expected molecular ion at *m*/*z* 406 while the methyl ester derivative produced the molecular ion at *m*/*z* 347.9 (348). Both molecular ions exhibited four chlorine isotope patterns in their mass spectra. GC-high resolution (HR)–EI-MS experiment was performed on the TMS derivative to determine the element composition of compound **2**. A measured value of 405.9488 was obtained which was within 5.84 ppm of the calculated value for $C_{16}H_{14}O_2Cl_4Si$. Thus, the elemental composition for component **2** was suggested to be $C_{13}H_6O_2Cl_4$.

3.4. NMR analysis of 1a, 1b and 2 extracted from Rehau Rau-SIK silicone tubing

Due to the limited quantity of the sample isolated, only proton NMR spectra were obtained. These spectra were consistent with the structures shown below. Each spectrum has five resonances in the aromatic region, consistent with five aromatic protons on two rings. Three of the five aromatic protons on one of the rings have the coupling pattern of a 1,2,4 tri-substituted benzene ring. The other two protons are ortho to each other (J = 8.4 Hz) in compound **1a**, meta to each other (J = 1.9 Hz) in compound **1b** and para to each other (J < 0.5 Hz) in compound **2**. The assignments of the Proton NMR spectra are summarized in Table 5.

Table 5
^{1}H NMR chemical shifts of compounds 1a, 1b and 2.

Position	1a	1b	2
3′	7.83, d, J = 2.2 Hz	7.75, d, J = 2.1 Hz	7.79, d, J = 2.1 Hz
5′	7.58, dd, J = 2.2, 8.3 Hz	7.50, dd, J = 2.1, 8.2 Hz	7.55, dd, J = 2.1, 8.3 Hz
6′	7.42, d, J = 8.3 Hz	7.35, d, J = 8.2 Hz	7.44, d, J = 8.3 Hz
3	–	7.81, d, J = 1.9 Hz	7.91, s
5	7.71, d, J = 8.4 Hz	7.47, d, J = 1.9 Hz	–
6	7.84, d, J = 8.4 Hz	–	7.75, s

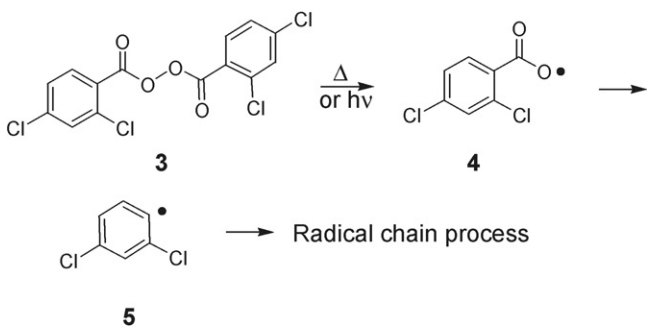
3.5. Manufacturer review

On discovery that the additional peaks were leaching from the Rehau RAU-SIK silicone tubing, Rehau was contacted to obtain further information. They indicated two potential low level leaching impurities 2,4-dichlorobenzoic acid and 2,4-dichlorophenol. 2,4-Dichlorobenzoic acid is a breakdown product of the peroxide catalyst, bis(2,4-dichlorobenzoyl) peroxide, used to propagate cross-linking of the silicone gel during the tubing manufacturing process. The post-curing process used in tubing manufacture removes the 2,4-dichlorobenzoic acid to low levels through heat sublimation. In our laboratory studies, however, we did not identify the leachables as being either of these compounds. The manufacturer also confirmed that the same manufacturing process is used for both technical (RAU-SIK) and medical (RAUMEDIC-SIK) grade peroxide-cured tubing.

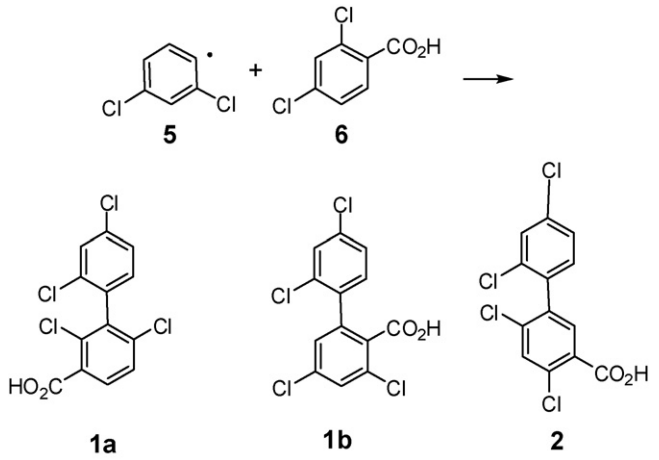
3.6. Synthesis, purification and structure confirmation of Rehau Rau-SIK extractables

The presence of compounds **1a**, **1b**, and **2** in the silicone rubber tubing and their proposed structures are consistent with the free radical chemistry used to polymerize silicone monomers. The polymerization process used to produce the tubing employs the peroxide catalyst bis(2,4-dichlorobenzoyl) peroxide (**3**, Scheme 1) to initiate the free radical chain reaction. Substituted peroxides such as this are widely used as initiators in organic synthesis and polymer chemistry. Under thermal or photochemical conditions, the peroxide bond of **3** is homolytically cleaved to produce two acyl radicals (**4**). The acyl radicals decarboxlyate to form two phenyl radicals (**5**), which initiate the radical chain process with the silicone monomer.

Since the silicone monomer is present in large excess compared with the radical **5** and other components in the reaction mixture, the predominant reaction of **5** is with the monomer. However, the radical **5** can react with other substrates present in the reaction mixture. Specifically, 2,4-dichlorobenzoic acid (**6**), present either due to quenching of the radical **4** or as an impurity in the peroxide, can react at its three unsubstituted positions with **5** to form the observed products (Scheme 2). The formation of biaryl compounds by the homolytic cleavage of peroxides in this manner is in fact a well-known synthetic transformation [1]. The presence of compounds **1a**, **1b** and **2** in the silicone rubber tubing and their proposed structures are fully consistent with the known radical chemistry of peroxides.



Scheme 1.



Scheme 2.

To confirm by HPLC chromatography that the isolated compounds from the synthetic route were the same as the extractable impurities in Intron samples, **1a**, **1b** and **2** were co-injected with an Intron sample, onto a Vydac C18, 4.6 mm × 150 mm, 5 μM column and eluted with a ACN and 0.1%TFA 0 → 90% gradient. The compounds co-eluted with the extractables in the Intron sample. They were also spiked with purified extractable impurities **1a**, **1b** and **2** from Rehau RAU-SIK silicone tubing and their co-elution confirmed their identity. They were further analyzed by mass spectrocopy and ^{1}H and ^{13}C NMR spectroscopy. The HPLC response factor was determined for compound **2** based on the weight of a sample injected in the HPLC. A response factor correction of 8.2 was calculated.

3.7. Mass spectral analysis of synthetic 1a, 1b and 2

The three synthesized compounds, **1a**, **1b** and **2**, were analyzed by MS for structure characterization. The ESI mass spectrum of each of these samples exhibited an abundant $(M-H)^-$ ion at m/z 333. The EI spectrum of each of these samples produced molecular ion at m/z 333.9 (334) and each molecular ion was consistent with a four chlorine isotope pattern. HR–EI-MS experiments were performed to establish the elemental composition of each compound. The three measured values, 333.9078, 333.9117 and 333.9081, for **1a**, **1b** and **2** respectively were consistent with the elemental composition of $C_{13}H_6O_2Cl_4$.

3.8. NMR analysis of synthetic 1a, 1b and 2

Proton and carbon NMR spectra of the three compounds were consistent with the structures. Proton NMR spectra were identical to those of the samples isolated from the silicone tubing. Carbon NMR spectra of the three compounds were also very similar to each other. They all have a resonance at around 166 ppm that can be assigned to the COOH group. They all have twelve additional resonances (five protonated and seven non-protonated carbons) in the region between 125 and 140 ppm those can be assigned to aromatic carbons.

Two-dimensional HMBC spectra were obtained for the three compounds to provide additional structural information. These spectra provide connectivity between proton and carbons that are two or three bonds apart. The HMBC spectra were consistent with the structures of the compounds. For example, a correlation was observed between H6 and C7 for compound **1a**. A correlation was

observed between H6 and C7 for compound **2**. No correlation was observed between C7 and any proton for compound **1b** due to the fact that no proton is two or three bonds away from C7. Two-dimensional NOESY spectrum was obtained for compound **2** and NOE was observed between H6' and H6 as expected.

4. Conclusion

Low levels of extractables have been observed in Intron finished product vials following periods of filling line down time. Laboratory studies and information from the manufacturer confirmed the RAU-SIK silicone tubing as the source of the extractable impurities. They were identified as three structural isomers derived from the reaction of dichlorobenzoic acid and dichlorobenzene radical. These three products have also been observed in Rehau RAUMEDIC-SIK silicone tubing (medical grade silicone tubing), widely used for dialysis, infusion and ECC (exocorporal circulation).

Assessment of the potential toxicity of the extractables concludes that their detection in batches of Intron does not represent a risk for patients under the conditions of clinical use. Thus, there was no concern with the safety and efficacy of marketed product. A new type of silicone tubing, Pt cured RAUMEDIC-PLATIN-SIK tubing, was obtained from Rehau and did not have these leachable impurities.

A final important point in the current discussion for an overall risk assessment of the impurities is that of actual concentration in the final drug product. The available data demonstrated that the concentration of these impurities is time- and tubing age-dependent. In the large majority of time during the manufacturing process where solution is simply flowing freely through the tubing to vials, the impurities would be expected to be present but below detectable levels. In those small number of vials which are filled immediately after start up of a line or after a where the filling solution has been in extended contact with the tubing, the concentration of impurities would be expected to rise in direct proportion to the time of the shut down. In fact, in the four samples analyzed that were "enriched" by permitting prolonged (>30 min) contact of the solution with the tubing in the present investigation, the maximum amount of these impurities ranged from 219 to 551 ng for the total of the three impurities with the individual impurities ranging from 58 to 192 ng per vial of drug. These levels can be considered representative of the amount of these impurities that may be anticipated at the start up of a run or after a shut down with fresh tubing. This represents a dose of less than or equal to 11 ng/kg body weight for total impurities or less than or equal to 4 ng/kg for each individual impurity (based on a 50-kg patient) and would be achieved in a very small number of vials from a usual batch.

Leaching of impurities from the reactants, packaging containers or even labels is not uncommon. In a recent study Maus et al. [2] showed detection and identification of volatile byproducts acetophenone and 2-phenyl-2-propanol, in solid oral compressed tablets. These impurities leached from common polymer cross-linking agent dicumyl peroxide, present in the cross-linked foam pads used as packing material, to transport in bulk containers prior to packaging. In another similar study Brooks and co-workers [3] showed a low level degradate of famotidine in film-coated packaged in child-resistant (CR) foil pouches which were stressed at 40 °C/75% relative humidities (RH) for 4 months. A detailed analysis of mass spectrum of the protonated degradate ion in a LC–MS–MS study indicated that the carbon was added to the side of N-(aminosulfonyl)-propanimid-amide of famotidine.

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