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Preparation and characterisation of hexamidine salts

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Graphical abstract

a) Hexamidine diisethionate b) Hexamidine dihydrochloride

Abstract

Hexamidine diisethionate (HEX D) has been used in the personal care industry and in a number of over-the-counter (OTC) drug products as an antimicrobial agent since the 1950's. Recently, the compound has also been investigated for its beneficial effects on skin health. Surprisingly, there is only limited information describing the physicochemical properties of this compound in the literature. The

objective of this work was therefore to conduct a comprehensive programme of characterisation of HEX D as well as its dihydrochloride salt (HEX H). HEX H was prepared from HEX D by a simple acid addition reaction. Both salts were characterised using Nuclear Magnetic Resonance (NMR), Differential scanning calorimetry (DSC), and Thermogravimetric analysis (TGA). A new high performance liquid chromatographic method was developed and validated for both compounds. The pH in aqueous solution as well as respective distribution coefficients between octanol and pH 7.4 buffer were also determined. Finally, solubility and short term stability studies were conducted in a range of solvents. NMR analysis confirmed the preparation of HEX H from HEX D. Thermal analysis indicated the melting points of HEX D and HEX H were 225°C and 266°C respectively. HPLC analysis confirmed the purity of both salts. Log D values at pH 7.4 were -0.74 for HEX D and -0.70 for HEX H respectively. The physicochemical properties of two HEX salts have been established using a range of analytical approaches. Detailed solubility and stability data have also been collated. This information will be useful in the design of novel formulations for targeted delivery of these compounds to the skin.

Key words: Hexamidine, salts, characterisation, preparation, pre-formulation, delivery

1. Introduction

Hexamidine (HEX) is an aromatic diamidine and a strong organic base. Although primarily used as the diisethionate salt (HEX D), it was firstly synthesised as the dihydrochloride (HEX H) and patented by Ewins et al. (1939) for May & Baker Limited (U.K.). The company was interested in the trypanocidal activity of the diamidines and the dihydrate of HEX H was subsequently demonstrated to be the most potent of the group (Ashley *et al.*, 1942). Antiprotozoal activity was demonstrated more than 50 years later when Brasseur *et al.* (1994) used HEX D to treat two subjects affected by *Acanthamoeba* keratitis. HEX D has also shown efficacy against *Pseudomonas aeruginosa*, *Proteus*, *Escherichia coli*, *Staphylococcus aureus* and *Tsukamurella paurometabolum* (van Ketel, 1975; Granel *et al.*, 1996). A more recent *in-vitro* study demonstrated HEX D efficacy against a series of multi-drug resistant gram-

positive bacteria (Grare *et al.*, 2010). Geratz *et al.* (1973) demonstrated the efficacy of HEX H dihydrate as an enzyme inhibitor with K_i values of 1.9, 4.5 and 7.4 μM, trypsin, pancreatic kallikrein and thrombin respectively. Enyedy *et al.* (2001) confirmed HEX inhibitory activity against thrombin (K_i value 224 nM) and matriptase (K_i = 924 nM), but did not specify if the active was used as the free base or salt. Finally, an *in-vivo* study investigated the effect of two HEX salts on nitric oxide synthase (NOS). Surprisingly, while HEX D significantly decreased NOS activity, the tetrachloroplatinate (II) salt had no effect on NO generation (Morgant *et al.*, 1998).

A number of publications have focussed on the role of HEX as an anti-aging and moisturising active in cosmetics and specifically the influence of HEX on various biomarkers of corneocyte maturity and skin turnover. Kimball *et al.* (2012) speculated that HEX might attenuate the skin ageing process because of its inhibitory activity on serine proteases associated with skin inflammation. Both skin inflammation and abnormal lipid biosynthesis have been linked to skin ageing (McGrath *et al.*, 2012). Osborne *et al.* (2009) and Jarrold *et al.* (2010a) showed that when human skin equivalent cultures were exposed to HEX, cholesterol, fatty acid and sphingolipid biosynthesis as well as cholesterol and fatty acid uptake were downregulated while cholesterol efflux was upregulated. Jarrold *et al.* (2010b) demonstrated that the application of a cosmetic moisturiser containing HEX, niacinamide and palmitoyl-lysine-threonine significantly increased the number and size of mature corneocytes of the facial stratum corneum of twenty female subjects. Significant thickening of the stratum corneum (SC) as well as a reduction in transepidermal water loss of the volar forearm was reported for 36 female subjects following treatment with a cream containing HEX and niacinamide (Kaczvinsky *et al.*, 2010). However these *in vivo* studies did not specify if the active was used as the free base or salt.

The safety of HEX and HEX D has been assessed by the Cosmetic Ingredient Review Expert Panel (2007). The panel concluded that both actives are safe when used in cosmetics at concentrations less than or equal to 0.10%. This opinion was subsequently confirmed by the European Parliament and the Council of the European Union (2009) which fixed the maximum allowed concentration of HEX and its salts in cosmetic products to 0.10%. However, several cases of allergic contact dermatitis have

been reported since HEX has been in use (Gougerot *et al.*1950; Sidi *et al.*, 1969; van Ketel, 1975; Robin,1978; Dooms-Goossens *et al.* 1989; Brand and Ballmer-Weber, 1995; Mullins, 2006;).

To date, HEX D has been used as a preservative in ~40 cosmetic products and in a number of over-the-counter formulations (Cosmetic Ingredient Review Expert Panel, 2007). Surprisingly, there is only a limited amount of information describing the physicochemical properties of HEX in the literature (British Pharmacopoeia, 2015). The use of HEX H as an alternative salt to HEX D has also not been explored. The objective, therefore, of the present work, was to undertake a comprehensive programme of characterisation of HEX D and HEX H. In the longer term this information should assist in the design of formulations which target this active more effectively to the skin.

2. Materials and Methods

2.1 Materials

HEX D (Laboratoires Sérobiologiques, France) was a gift from Procter & Gamble (U.S.A.), while HEX H was synthesized and purified in-house. Propylene glycol, polyethylene glycol 200, HPLC grade isopropyl alcohol, trifluoroacetic acid (HPLC grade) and absolute ethanol were supplied by Fisher Scientific (U.K.). HPLC grade solvents (acetonitrile, methanol, water), glycerol, isopropyl myristate, 1-octanol, 2-ethylhexyl salicylate, 1 M hydrochloric acid solution and dimethyl sulfoxide-d_6 were provided by Sigma-Aldrich (U.K.). Dimethyl sulfoxide was supplied by VWR International (U.K.). Propylene glycol monolaurate, Labrafac™ PG and Transcutol® P were received as gifts from Gattefossé (France). 1,2-pentanediol was provided by Surfachem Group (U.K.). Dimethyl isosorbide (Arlasolve®) was supplied by Croda International (U.K.). Oleic acid was provided by Fluka (U.K.). Miglyol® 812 N was supplied by Sasol (Germany). Dipropylene glycol was provided by Acros Organics (Belgium). Phosphate buffered saline was prepared using Dulbecco A tablets (Oxoid, U.K.).

2.2 Methods

Conversion of HEX D to HEX H

Approximately 50 mL of 1 M hydrochloric acid solution were heated at 100 ± 1°C using an Ikamag® C-MAG HS 7 magnetic stirrer ceramic heating plate (IKA, Germany) equipped with an ETS-D5 electronic contact thermometer (IKA, Germany). HEX D was dissolved in the solution followed by stirring of the mixture and cooling (15 min). The flask was subsequently placed on ice for 30 min to allow recrystallisation of the product. Finally, crystals were recovered by means of vacuum filtration and dried at room temperature. Hydrogen-1 and carbon-13 nuclear magnetic resonance (^{1}H and ^{13}C NMR) spectroscopy were used to confirm the structure of the starting material and the product of the reaction. All spectra were acquired in dimethyl sulfoxide-d_6 on a Bruker Avance 400 MHz NMR spectrometer (Bruker Corporation, U.S.A.) and processed using MestReNova® 9.0.1 (Mestrelab Research, Spain).

Thermal analysis

The melting points of HEX D and HEX H were examined using thermogravimetric analysis (TGA) and differential scanning calorimetry (DSC). TGA was performed using a Discovery TGA (TA Instruments, U.S.A.) system. Each active was weighed in an open aluminium pan (TA Instruments, U.S.A.) and then heated inside the Discovery TGA furnace. The starting temperature and the final temperature were set to 25°C and 400°C, respectively, while the heating ramp was 10°C/min. A nitrogen flow of 25 mL/min was supplied throughout the analysis in order to create an inert atmosphere around the sample. A DSC Q2000 (TA Instruments, U.S.A.) system was used for the DSC analysis. Each active was weighed in a hermetic aluminium pan (TA Instruments, U.S.A.) which was subsequently sealed with a hermetic aluminium lid (TA Instruments, U.S.A.) using a Tzero™ press (TA Instruments, U.S.A.). An empty hermetic aluminium pan (sealed with a hermetic aluminium lid) was used as a reference. Both the sample and reference were heated from 40°C to 290°C, with a heating ramp of10°C/min and a nitrogen flow of 50 mL/min.

UV, HPLC analysis and method validation

A Spectronic BioMate™ 3 UV/VIS spectrophotometer (Thermo Scientific, U.S.A.) was used to carry out an UV scan of a solution of each active in HPLC grade water. The UV absorption spectrum was acquired between 200 and 300 nm (step = 1 nm) in order to identify the wavelength at which the absorption of light was specifically due to each active. The HPLC system consisted of a Hewlett-Packard (U.S.A.) series 1100 quaternary pump, an Agilent Technologies (U.S.A.) series 1100 autosampler, a Hewlett-Packard (U.S.A.) series 1100 system controller, an Agilent Technologies (U.S.A.) series 1100 degasser and an Agilent Technologies (U.S.A.) series 1100 UV detector. ChemStation® Rev.A.09.03 (Agilent Technologies, U.S.A.) software was used to analyse the data. HEX D was analysed with a Luna® 5 µm C_8 150 × 4.60 mm reversed phase column (Phenomenex, U.K.) equipped with a universal HPLC guard column (Phenomenex, U.K.) packed with a SecurityGuard™ C_8 cartridge (Phenomenex, U.K.). The mobile phase consisted of 75% v/v HPLC grade water (0.1% v/v HPLC grade trifluoroacetic acid) and 25% v/v HPLC grade acetonitrile. A Capcell Pak® MGIII 5 µm C_{18} 250 × 4.60 mm reversed phase column (Shiseido, Japan) was used to analyse HEX H. A universal HPLC guard column (Phenomenex, U.K.) packed with a SecurityGuard™ C_{18} cartridge (Phenomenex, U.K.) was attached to the column. The mobile phase consisted of 72% v/v HPLC grade water (0.1% v/v HPLC grade trifluoroacetic acid) and 28% v/v HPLC grade acetonitrile. For both HEX D and HEX H, the UV detector was set to 261 nm, the flow rate to 0.7 mL/min and the column temperature to 35°C. The injection volume was set to 10 µL for HEX D and 20 µL for HEX H. Linearity, specificity, accuracy, precision, lower limit of detection (LOD) and lower limit of quantification (LOQ) of both methods were validated according to International Conference on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use (2005).

pH and log $D_{o/w}$ determination

All pH measurements were taken using a SympHony® SB70P pH meter (VWR International, U.K.) at 25 ± 1°C. Four solutions of each active in deionised water were tested (0.001, 0.01, 0.1 and 1

mM) with the pH of deionised water taken as the control. The method used to measure the log $D_{o/w}$ of HEX D and HEX H was adapted from OECD guidelines (2006). 1-octanol was mutually saturated with PBS (pH = 7.4 ± 0.5 at 25°C) by slow-stirring for 48 h at 25 ± 1°C. The system was allowed to equilibrate in a separation funnel for 24 h. Two solutions of known concentrations of HEX D or HEX H in PBS saturated with 1-octanol (pH = 7.4 ± 0.5 at 25°C) were prepared. Solutions were mixed in different proportions (1:1, 2:1 and 1:2) with 1-octanol saturated with PBS (pH = 7.4 ± 0.5 at 25°C), placed in glass test tubes sealed with Parafilm® and allowed to rotate on a rotor for 24 h at 25 ± 1°C. The two-phase systems were then left to stand and equilibrate for 48 h at the experimental temperature. At the end of the equilibration period, both phases were sampled with dilution where necessary. Amounts of HEX D and HEX H were measured by HPLC and used to calculate the log $D_{o/w}$ (pH = 7.4) as follows:

$$\log D_{o/w} = \log \frac{[\text{Active}_{\text{octanol}}]}{[\text{Active}_{\text{water (pH = 7.4)}}]} \quad \textbf{(Equation 1)}$$

Solubility and stability studies

For solubility determination an excess amount of active was added to each solvent in a glass test tube containing a Teflon®-coated magnetic stir bar. The test tube was sealed with Parafilm® and placed in a SUB 28 thermostatically controlled water bath (Grant Instruments, U.K.) equipped with a Telesystem HP 15 submersible magnetic stirrer (Variomag®-USA, U.S.A.). The system was allowed to stir and equilibrate for 48 h at 32 ± 1°C to obtain a saturated solution. After the 48 h period, a sample was withdrawn from the test tube and centrifuged at 13200 rpm for 15 min at 32 ± 1°C in an Eppendorf 5415R centrifuge (Eppendorf, Germany). Finally, the supernatant was suitably diluted and the concentration of the active was determined by HPLC. Stability of HEX D and HEX H in several solvents and binary solvent systems was investigated for 120 h at 32 ± 1°C. A solution of known concentration of active was prepared and placed in a screw top glass test tube with a stir bar. The sample was sealed

and allowed to stir for 120 h at 32 ± 1°C as for solubility studies and aliquots were removed at 0, 24, 48, 72, 96 and 120 h. Following sample dilution the concentration of the active was determined by HPLC.

3. Results and discussion

Conversion of HEX D to HEX H

The hypothetical double displacement reaction between HEX D and HCl is shown in Equation 2.

$$C_{20}H_{26}N_4O_2(C_2H_6O_4S)_2 + 2HCl \rightarrow C_{20}H_{26}N_4O_2(HCl)_2 \downarrow + 2C_2H_6O_4S \quad \textbf{(2)}$$

The ^{1}H NMR spectrum of HEX D in dimethyl sulfoxide-d_6 is shown in Figure 2a. The dimethyl sulfoxide-d_6 quintuplet at 2.50 ppm was used as a reference to scale the x-axis of the spectrum. A water singlet at 3.30 ppm reflects the hygroscopicity of dimethyl sulfoxide-d_6 which readily absorbs moisture from the atmosphere and glassware (Gottlieb *et al.*, 1997).

Figure 2a shows two triplets at 2.68 and 3.65 ppm which are assigned to the methylene hydrogens of the isethionate anion (g and h respectively), while the singlet at 4.47 ppm is assigned to the hydroxyl group (i). Interestingly, these peaks are not present in the ^{1}H NMR spectrum of the HEX H crystals, while those for the HEX moiety of the molecule are evident (Figure 2b). In Figure 2b, the water signal is more intense than for Figure 2a and cannot be attributed solely to the moisture absorbed by the dimethyl sulfoxide-d_6. This strong signal may reflect residual aqueous reaction medium or water of crystallisation which becomes trapped inside the crystals during the recrystallisation process. Further confirmation of HEX H as the product is provided by comparison of the ^{13}C spectrum of HEX D with that of HEX H (Figures 3a and 3b). The DMSO-d_6 septuplet at 39.52 ppm was used as a reference to scale the x-axis of both spectra. Two singlets at 53.69 and 57.66 ppm (Figure 3a) are assigned to the methylene carbons of the isethionate anion (9 and 10 respectively). Again, these peaks are not present in the ^{13}C NMR spectrum of the reaction product (Figure 3b).

Thermal analysis

The results of the TGA and the DSC analysis of HEX D are shown in Figure 4. TGA is a well-established method for the characterisation of materials and is particularly useful in determining loss of water molecules and compound degradation temperatures (Coats and Redfern, 1963). There is no weight loss of HEX D between 25°C and 290°C (Figure 4a). However, degradation occurs between 300°C and 375°C and only ~ 12% of the initial weight of HEX D remains at 400°C. For DSC analysis, two endothermic events were observed; the first has an onset temperature of 176.62°C and the second is 224.86°C. It may be hypothesised that these two peaks reflect the melting of two different crystal structures of HEX D. Considering that pentamidine diisethionate, the lower homologue of HEX diisethionate, exists in at least four crystalline forms (Steele, 1990; Chongprasert *et al.*, 1998), the possibility of multiple polymorphs of HEX D was expected. Fucke *et al.* (2008) identified ten anhydrous and two dihydrate polymorphic forms of HEX D. Furthermore, the authors confirmed that HEX D does not directly melt but undergoes a phase transition (Personal communication Fucke, 2015). This suggests that the first endotherm in Figure 4a is the phase transition from a low-temperature form to the stable high-temperature crystal form which melts at 224.86°C. The corresponding results for DSC and TGA analyses of HEX H are shown in Figure 4b.

HEX H exhibits 11.1% weight loss between 25°C and 100°C and single-stage degradation between 265°C and 350°C. Thus only ~7% of the initial weight of HEX H remains at 400°C. The initial weight loss may be attributed to the evaporation of water from the sample. This is consistent with the ^{1}H NMR spectrum of HEX H (Figure 3) and the presence of water of crystallisation. The water content of 11.1% gives a stoichiometric ratio of three molecules of water per molecule of HEX H indicating the salt was recrystallised in its trihydrate form. DSC analysis of HEX H shows three endothermic events (Figure 4b). The first occurs between 40°C and 120°C, and represents the loss of water of crystallisation already observed in the TGA curve. The second peak has on onset temperature of 223.2°C. This value is very close to the melting point of the stable high-temperature crystal form of HEX D (Figure 4a). It might be speculated that this second endotherm was the melting of residual HEX D which was not

converted to HEX H and remains as an impurity at the end of the conversion reaction. Finally, the third sharp endothermic event with an onset temperature of 265.5°C is presumed to be the melting point of HEX H.

UV and HPLC analysis and method validation

HEX D and HEX H exhibited a suitable UV peak for analysis at 261 nm. For the HPLC analysis calibration curves (ranging from 0.5 µg/mL to 20 µg/mL) were constructed. The linearity for both methods was confirmed by the correlation coefficient (r^2) which was equal to 0.99 across the experimental range. There were no interfering peaks at the retention times of the analytes which were 5. 1 min for HEX D and 8.2 min for HEX H. Recovery of each compound within the range from 90% to 110% was achieved. In addition, the %RSD for the intra-day and inter-day precision were below 5% and 10% respectively, thus demonstrating the repeatability of the proposed methods. The LOD and LOQ for HEX D were 0.54 µg/mL and 1.64 µg/mL. The values obtained for HEX H, were 0.40 µg/mL for the LOD of and 1.21 µg/mL for the LOQ. These values are also lower than values previously reported for HPLC analysis of HEX D (Taylor *et al.*, 1983; De Bukanski and Masse, 1984).

pH in aqueous solution and log $D_{o/w}$ at pH = 7.4

Solutions of HEX D and HEX H in deionised water were as expected slightly acidic (pH ranging from 6.3 to 6.4). The log $D_{o/w}$ at pH = 7.4 and 25 ± 1°C and the recovery of HEX D and HEX H are reported in Table 1. Both actives showed a negative log $D_{o/w}$, with HEX D having a significantly lower value than HEX H (t-test, $p < 0.01$).

Solubility and stability

The solubility at 32 ± 1°C of HEX D and HEX H in a range of different solvents is shown in Figures 5a and 5b. Data for solvents in which both actives had solubility > 1 mg/mL are pooled in Figure 5a

while those in which they had solubility < 1 mg/mL are presented in Figure 5b. The only exception to this is TC which is included in both figures. HEX H, in fact, had a solubility of 2.00 mg/mL in TC while the value for HEX D was only 0.37 mg/mL.

Both actives exhibited highest solubility in DMSO compared with all the other solvents studied; both actives were also soluble in PG, glycerol and methanol, sparingly soluble in 1,2-PENT and PEG 200 and only slightly soluble in PBS, ethanol, DPG and TC (HEX H only). In addition, HEX D was soluble in water, while HEX H was only sparingly soluble in water. The solubility of HEX D and HEX H in water was fifteen and ten times, respectively, higher than that in PBS (pH = 7.4). Considering that the pH of water was 6.36 and that the pK_a of the amidino group of HEX is 11, the increase in pH resulted in a lower ionisation and, as a result, in a lower solubility of the actives in PBS. This effect of pH on solubility is commonly accepted and Avdeef (2007) has recently reviewed how it affects sparingly soluble ionisable drugs. The presence of other ions and components of the buffer is also expected to influence the solubility values obtained. For example, it is possible that phosphate anions may interact with hexamidine cations, precipitate them and reduce hexamidine concentration in solution. As no information is available in the literature on phosphate salts of HEX this is an area which deserves further investigation.

With the exception of TC and IPA, both HEX D and HEX H were practically insoluble in all other solvents studied. HEX H in particular, was so poorly soluble in 2-EHSAL, IPM and LABR that its solubility was below the LOQ (1.21 µg/mL) for HPLC analysis. The percentage of HEX D recovered after 24, 48, 72, 96 and 120 h at 32 ± 1°C in a series of solvents and selected binary solvent systems is shown in Table 2. The results summarised in Table 2 indicate that HEX D exhibits some instability in water. At 24 h recovery was 86.1 ± 3.6 % but there was no further degradation. Conversely, HEX D did not undergo degradation in the other solvents and binary solvent systems tested. Less than 8 % loss was observed after 120 h in PBS, PG, PEG 200, glycerol, PG:PGML (50:50) and DMSO:Methanol (50:50).The results of the stability studies of HEX H in the same solvents and binary solvent systems seen for HEX D are presented in Table 3. HEX H did not show any stability issues (Table 3) and less than 5 % loss was

observed after 120 h in water, PBS, PG, PEG 200, glycerol, PG:PGML (50:50) and DMSO:Methanol (50:50).

4. Conclusions

The selection of an active ingredient and the characterisation of its physicochemical properties is arguably the most important stage in the preformulation design of a topical. All available information about HEX and its salts was identified and reviewed. Although HEX D is the active that is currently used in personal care and pharmaceutical formulations, its dihydrochoride salt, HEX H also appears to be a suitable candidate molecule for delivery to the skin. We have confirmed that HEX H has a lower MW than HEX D but a higher melting point. Thermal analysis also confirmed that HEX D exists in different crystal forms and revealed that HEX H had recrystallised as a trihydrate during the conversion process. The measurement of the pH of the solutions of HEX D and HEX H in deionised water demonstrated that both salts are very weakly acidic. New HPLC analytical methods for the quantification of HEX D and HEX H were developed and validated. The solubility of HEX D and HEX H was studied in 19 solvents and both actives were found to be more soluble in those solvents having polar properties. The stability of HEX D and HEX H in solution and in a limited number of combinations of selected excipients was also evaluated. Overall, the findings are expected to be useful in the rational design of new formulations for both actives.

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Figure 4. TGA and DSC analysis of (a) HEX D and (b) HEX H

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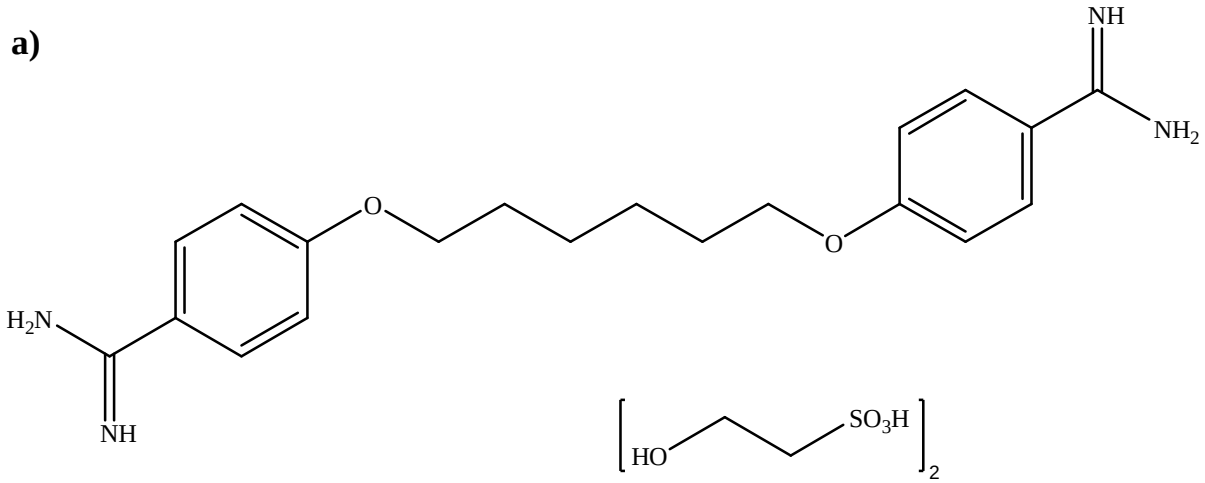
Figure 5. Solubility data for HEX D and HEX H at 32°C (a) Solubility > 1mg/mL (b) Solubility < 1mg/ml with exception of Transcutol™ which is shown in both figures (n≥ 3; Mean±S.D.)

a)



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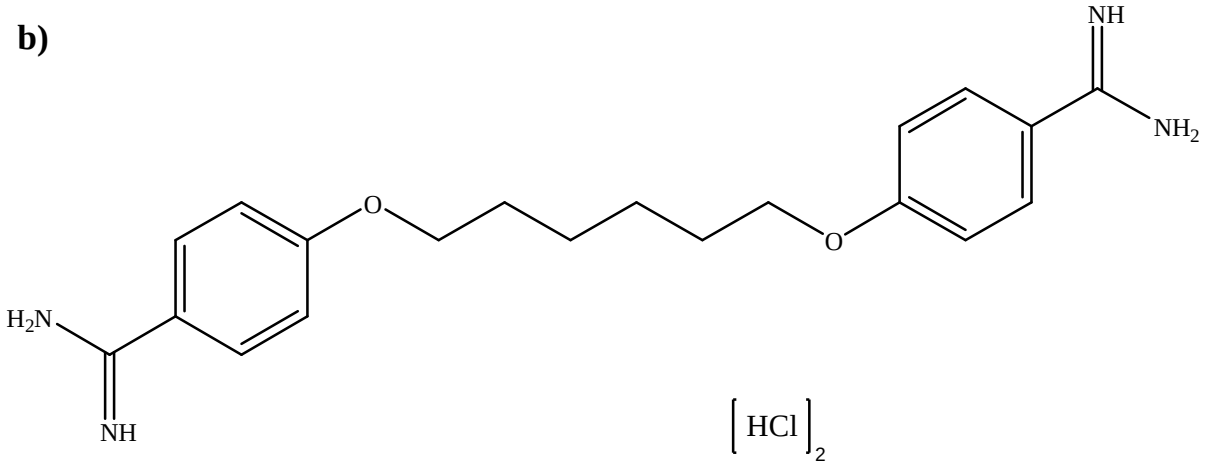

Figure 1

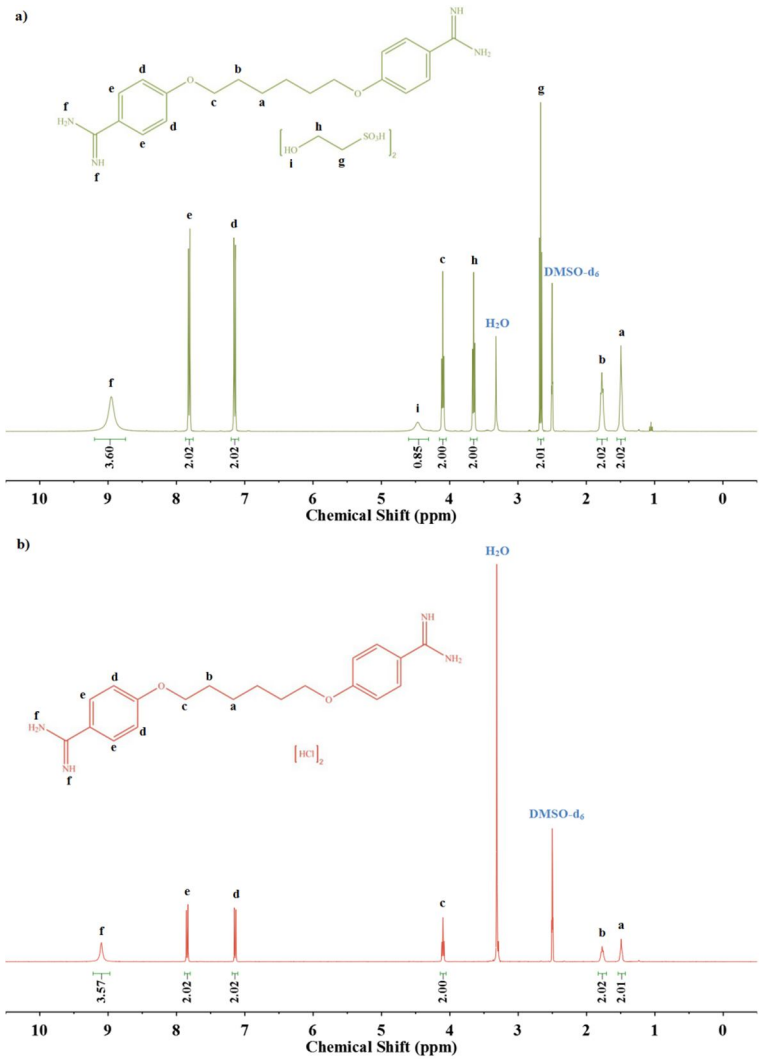

Figure 2

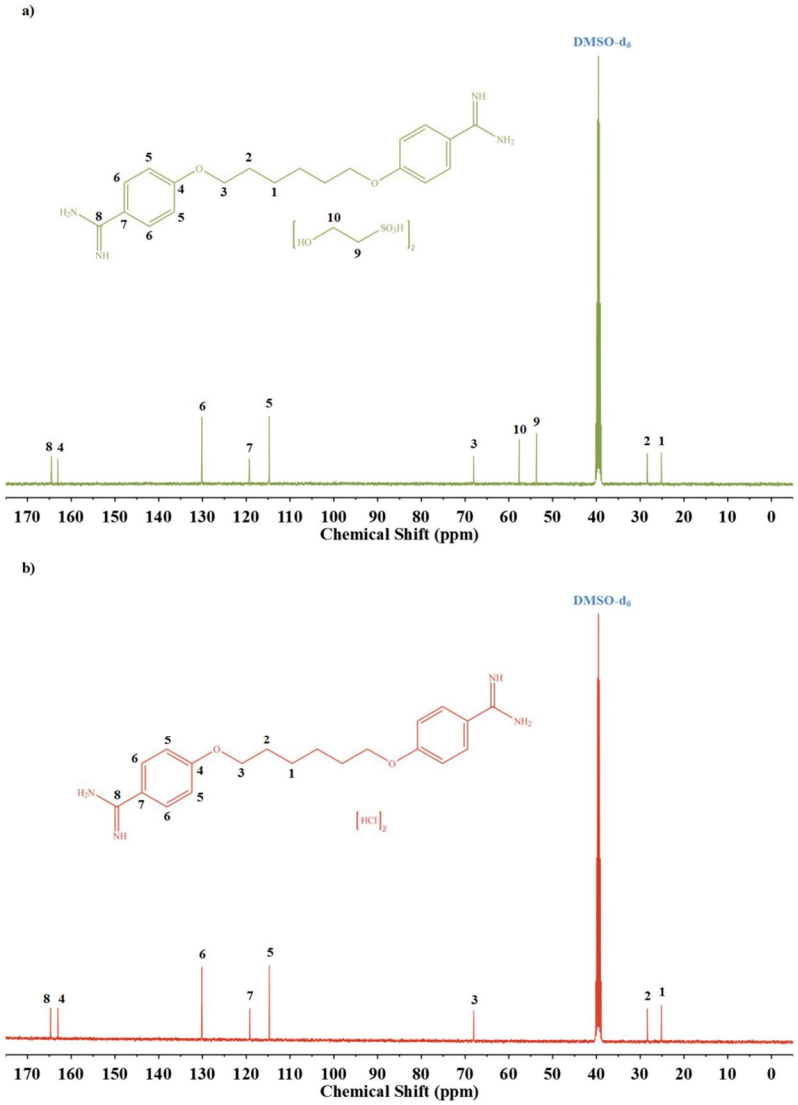

Figure 3

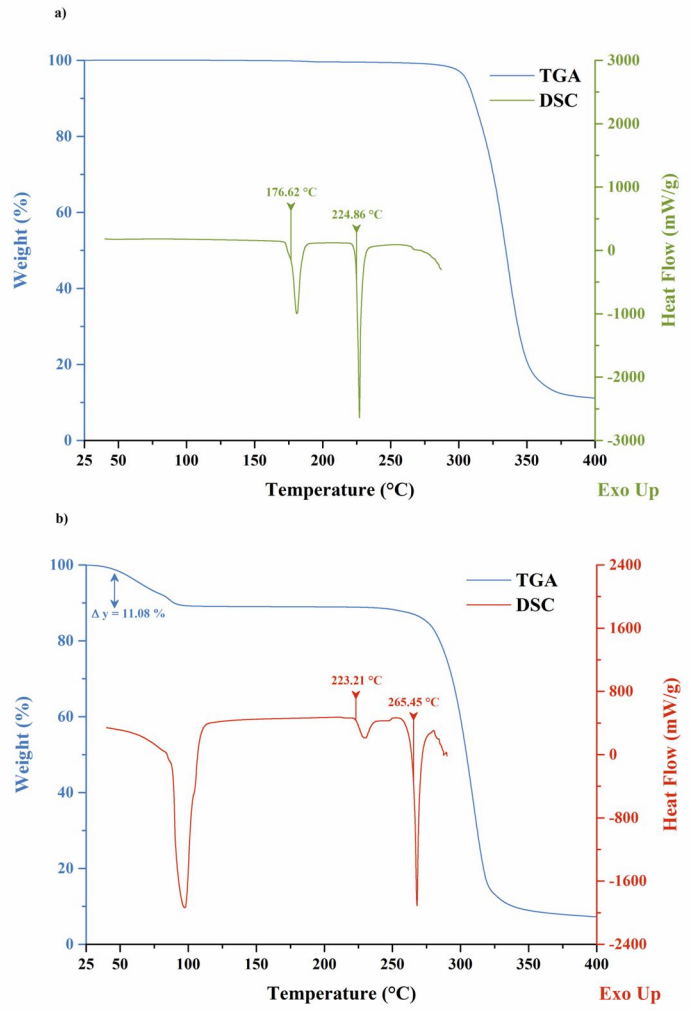

Figure 4

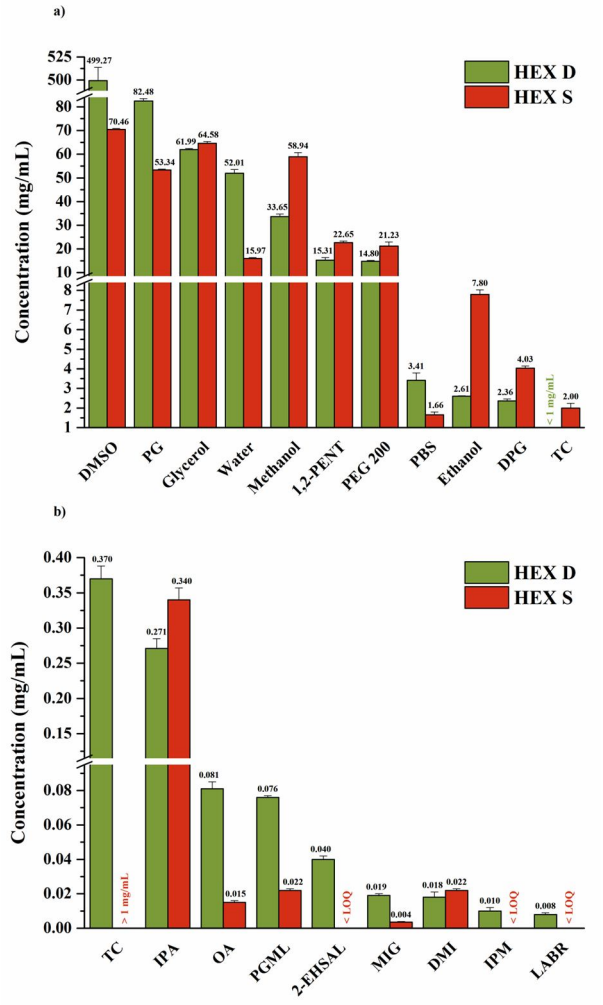

Figure 5

Table 1 Log $D_{o/w}$ at pH = 7.4 and 25 ± 1°C and recovery of HEX D and HEX H (n=9; mean ± SD)

Active	log $D_{o/w}$	Recovery (%)
HEX D	− 0.74 ± 0.02	101.2 ± 2.7
HEX H	− 0.70 ± 0.02	101.5 ± 1.6

Table 2. Recovery (%) of HEX D in a series of solvents and binary solvent systems after 24, 48, 72, 96 and 120 h at 32 ± 1°C (3≤n≤4; mean ± SD)

Time (h)	Water	PBS	PG	PEG 200	Glycerol	PG:PGML (50:50)	DMSO:Methanol (50:50)
0	100.0	100.0	100.0	100.0	100.0	100.0	100.0
24	86.1 ± 3.6	99.27 ± 4.42	99.83 ± 1.96	94.36 ± 2.34	97.64 ± 2.83	94.78 ± 5.83	93.57 ± 2.34
48	80.7 ± 6.5	100.3 ± 4.5	103.3 ± 2.9	91.8 ± 7.8	98.4 ± 3.1	95.1 ± 4.2	99.3 ± 1.7
72	83.7 ± 1.5	99.9 ± 2.5	98.8 ± 3.4	87.8 ± 3.6	99.5 ± 2.0	95.4 ± 1.6	98.5 ± 0.8
96	82.5 ± 3.8	100.3 ± 2.2	99.2 ± 3.7	92.4 ± 1.7	98.3 ± 4.0	93.8 ± 1.6	98.6 ± 1.8
120	82.6 ± 4.4	102.1 ± 1.6	104.0 ± 7.9	93.0 ± 4.2	98.3 ± 3.4	94.6 ± 3.1	102.1 ± 3.0

Table 3. Recovery (%) of HEX H in a series of solvents and binary solvent systems after 24, 48, 72, 96 and 120 h at 32 ± 1°C (n=4; mean ± SD)

Time (h)	Water	PBS	PG	PEG 200	Glycerol	PG:PGML (50:50)	DMSO:Methanol (50:50)
0	100.0	100.0	100.0	100.0	100.0	100.0	100.0
24	98.4 ± 1.8	101.2 ± 1.3	99.4 ± 2.5	99.6 ± 0.6	97.0 ± 0.3	101.5 ± 3.7	93.1 ± 2.3
48	99.7 ± 2.9	98.9 ± 1.5	101.1 ± 2.7	98.4 ± 1.2	101.0 ± 1.1	101.7 ± 2.6	96.3 ± 2.6
72	99.8 ± 2.7	100.3 ± 2.1	100.8 ± 2.7	98.5± 1.9	99.8 ± 2.1	99.9 ± 2.9	98.3 ± 1.9
96	100.0 ± 2.5	100.7 ± 0.2	100.6 ± 3.2	96.6 ± 2.5	99.5 ± 2.4	101.1 ± 4.3	97.2 ± 1.6
120	100.3 ± 1.9	99.1 ± 2.4	101.2 ± 2.1	95.1 ± 1.5	101.4 ± 2.4	99.6 ± 2.4	102.1 ± 1.3