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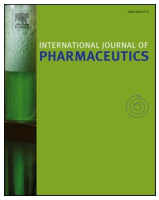

Characterisation of colloidal structures and their solubilising potential for BCS class II drugs in fasted state simulated intestinal fluid

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ABSTRACT

A suite of fasted state simulated intestinal fluid (SIF), based on variability observed in a range of fasted state human intestinal fluid (HIF) samples was used to study the solubility of eight poorly soluble drugs (three acidic drugs (naproxen, indomethacin and phenytoin), two basic drugs (carvedilol and tadalafil) and three neutral drugs (felodipine, fenofibrate, griseofulvin)). Particle size of the colloidal structures formed in these SIF in the presence and absence of drugs was measured using dynamic light scattering and nanoparticle tracking analysis.

Results indicate that drug solubility tends to increase with increasing total amphiphile concentration (TAC) in SIF with acidic drugs proving to be more soluble than basic or neutral drug in the media evaluated. Dynamic light scattering showed that as the amphiphile concentration increased, the hydrodynamic diameters of the structures decreased. The scattering distribution confirmed the polydispersity of the simulated intestinal fluids compared to the monodisperse distribution observed for FaSSIF v1). There was a large difference in the size of the structures found based on the composition of the SIF, for example, the diameter of the structures measured in felodipine in the minimum TAC media was measured to be 170 ± 5 nm which decreased to 5.1 ± 0.2 nm in the maximum TAC media point. The size measured of the colloidal structures of felodipine in the FaSSIF v1 was 86 ± 1 nm. However, there was no simple correlation between solubility and colloidal size.

Nanoparticle tracking analysis was used for the first time to characterise colloidal structures within SIF and the results were compared to those obtained by dynamic light scattering. The particle size measured by dynamic light scattering was generally greater in media with a lower concentration of amphiphiles and smaller in media of a higher concentration of amphiphiles, compared to that of the data yielded by nanoparticle tracking analysis.

This work shows that the colloidal structures formed vary depending on the composition of SIF which affects the solubility. Work is ongoing to determine the relationship between colloidal structure and solubility.

1. Introduction

The most common and preferred route of treatment is oral administration of solid drugs due to the straightforward and non-invasive nature of administration which aids in patient compliance. The drug material must pass through the gastrointestinal tract which is an intricate system where many factors control oral bioavailability including the solubility and dissolution of the drug material in the gastrointestinal environment. Gastrointestinal fluid is a complex media with many components whose concentration is variable based on individual and prandial state (Pyper et al., 2020).

Adequate drug solubility in the gastrointestinal tract is essential for systemic therapy of orally administered medications. In order to measure the solubility of poorly soluble drugs in vitro, simulated intestinal fluid (SIF) is used in place of human intestinal fluid (HIF). Typically a single FaSSIF (fasted state simulated intestinal fluid) is used (Dressman et al., 1998; Galia et al., 1998) which reflects average compositions of HIF rather than the full extent of variability in composition previously reported (Riethorst et al., 2016). An additional limitation of this material is absence of cholesterol in the composition of FaSSIF V1 and FaSSIF V2 (Dressman et al., 1998; Galia et al., 1998).

HIF is known to have a large variability in composition of its components which include numerous bile salts, phospholipids, fatty acids, cholesterol, products of lipid degradation and enzymatic secretion, and pH. Changes caused by postprandial state, secretions by the biliary and pancreas and also the ever-changing nature of the luminal area can further contribute to increased variability (Riethorst et al., 2016). Therefore, a suite of simulated intestinal fluids would better represent

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the intricate composition of HIF compared to a single media (e.g. FaSSIF V1) (Pyper et al., 2020).

Previous work reported by Khadra *et al.* (2015) found that six factors (pH, sodium taurocholate, lecithin, sodium phosphate, sodium chloride and sodium oleate) individually exert a statistically significant influence on drug solubility (Khadra et al., 2015). The most significant factor affecting acidic drugs' solubility was pH. The combination of pH and the concentration of solubilising amphiphile components in SIF (bile salt, lecithin and fatty acid) was significant for neutral and basic drugs (Perrier et al., 2018). It was found in this study that the amphiphilic components are roughly equal in terms of their solubilisation effects.

A recent study carried out by Abuhassan *et al.* investigated the solubility behaviour of twenty-one drugs in nine biorelevant simulated fasted intestinal media to identify if there were any correlations between the measured solubility and the drugs and drug classes (Abuhassan et al., 2022). The nine-point media used in this study was created by a multidimensional analysis to cover 90 % of the variability found fasted state HIF samples and covered a pH range from 5.72 to 8.04 and a [pH x total amphiphile concentration (TAC)] range of 15.07 to 122.4 (Pyper et al., 2020; Abuhassan et al., 2021). The acidic drugs analysed here were found to show a general increase in solubility that was primarily associated with their pKa and the pH of the media with limited impact of the total amphiphile concentration (TAC) in the media. The lowest and highest solubility measurements of the neutral and basic drugs was typically found in the lowest and highest [pH x TAC] media points, indicating a greater dependence on the TAC within the media (Abuhassan et al., 2022). The solubility of different compounds in HIF is associated with the colloidal structures that form by the various components in the intestinal fluid, e.g. micelles and vesicles of diverse morphologies (Parrow et al., 2020). However, there was no simple relationship between the composition and solubility which may indicate that colloidal structures and their interactions with drugs within the SIF also contribute to solubilisation.

Approaching the critical micelle concentration (CMC), bile salt molecules will begin to self-assemble into aggregates (Madenci and Egelhaaf, 2010). They can also form mixed colloids with phospholipids. At concentrations lower than the CMC, bile salts will permeate into phospholipid vesicles without disturbing the outer membrane and at concentrations greater than the CMC, mixed vesicles and mixed micelle structures will form (Parekh et al., 2023). The interactions between bile salts and phospholipids are controlled by the formation of hydrogen bonds between the head groups of the surfactants and the various hydrophilic regions of the salts which enable the correct orientation required for the arrangement of the mixed system (Parekh et al., 2023). Above their CMC, bile acids can solubilise various types of intestinal amphiphiles and lipids including phospholipids, fatty acids and cholesterol by forming mixed micelles (Wang et al., 2013). The resulting micelles and colloidal structures formed are able to increase the solubility of hydrophobic compounds which in turn can increase the concentration of dissolved drug (Clulow et al., 2017; Kleberg et al., 2010). De Smidt *et al.* showed that above the apparent sodium taurocholate CMC, the solubility of griseofulvin increases linearly with the concentration of bile salt (de Smidt et al., 1991). Here, they measured the CMC of bile to be 5.2 mM[15] which is considerably greater than the ~3 mM reported in other literature (Parekh et al., 2023; Clulow et al., 2017; McGown and Nithipatikom, 1988).

The various forms of colloidal structures present in SIF have been identified and characterised by a myriad of techniques. Microscopy techniques have been used such as optical microscopy, atomic force microscopy and transmission electron microscopy as well as scattering techniques including small-angle x-ray scattering, small-angle neutron scattering and dynamic light scattering (DLS) (Parrow et al., 2020; Parekh et al., 2023).

A study analysing FaSSIF versions 1, 2 and 3 (supplied by Biorelevant.com, London) used cryogenic transmission, electron microscopy to identify the colloidal structures present in each media. In FaSSIF-V1, various vesicles and micelles were found as well as agglomerates of micelles. In FaSSIF-V2, vesicles and micelles were detected with the most frequent form found was the thread-like micelle. The most common structure identified in the FaSSIF-V3 media was the disc shaped micelle, with other globular micelles and vesicles also present in the sample. The data showed that as the composition of the media were altered, the particle size and shapes of the colloidal structures formed were also altered (Klumpp et al., 2019). The colloidal structures of FaSSIF-V3 are different to those of versions 1 and 2 as a result of the different composition of FaSSIF-V3 as it includes glycocholate, lysophosphatidylcholine and sodium oleate (Fuchs et al., 2015). Both unilamellar and multilamellar vesicles were observed in samples of all three fasted state media (Klumpp et al., 2019).

Dynamic light scattering measurements recorded by Clulow *et al.* provided particle size analysis data of fasted state bile salt micelles, mixed micelles of bile salt/phospholipid/buffer and biorelevant media. Analysis of 5 mM sodium taurodeoxycholate in a 50 mM tris buffer formed micelles that were found to be 4.9 ± 0.9 nm (intensity distribution, diameter), mixed micelles of 5 mM sodium taurodeoxycholate/1.25 mM 1,2-dioleoyl-*sn*-glycero-3-phosphatidylcholine/50 mM tris buffer gave a size intensity distribution of 5.4 ± 0.9 nm (diameter) while the biorelevant FaSSIF media had a distribution of 48.0 ± 6.6 nm (Clulow et al., 2017). Dynamic light scattering carried out by Doak *et al.* of poorly soluble drugs found colloid formation in fed state simulated intestinal fluid. The BCS class II drug, itraconazole, was measured to have a solubilised micellar diameter of 129.0 ± 9.6 nm while the antiretroviral drug, delavirdine was determined to have a solubilised micellar radius of 125.6 ± 66.0 nm (Doak et al., 2010).

Nanoparticle tracking analysis (NTA) uses laser light scattering microscopy in combination with a camera that records the movement of the nanoparticles in solution. The primary advantage of using NTA compared to DLS, is that NTA has the ability to detect small and weakly scattering particles that are amongst larger, stronger scattering particles (Gallego-Urrea et al., 2011). The method by which DLS calculates particle size makes it very sensitive to the existence of larger particles which in turn means that the presence of dust particles or large particle aggregates can prevent accurate calculation of particle size (Filipe et al., 2010). NTA has been used for many different types of samples in a variety of fields including evaluating environmental samples and aggregates of nanoparticles and proteins (Gallego-Urrea et al., 2011), however, it has yet to be used to study simulated intestinal media.

The objective of this work was to use experimental data collected from solubility, dynamic light scattering (DLS) and nanoparticle tracking analysis (NTA) to better understand the relationship between enhanced solubility observed in simulated intestinal fluid and how this is affected by the nature and density of colloidal structures within the media.

2. Materials and methods

2.1. Materials

This study used six simulated intestinal fluid recipes; the minimum, Q1, median, Q3 and maximum [pH x total amphiphile concentration (TAC)] points from the work previously reported by Riethorst *et al.* (Riethorst et al., 2016) FaSSIF V1, from Biorelevant.com was used as a comparator within this study. The composition of these media are listed in Table 1. Sodium taurocholate (bile salt, BS), sodium oleate (free fatty acid, FFA) cholesterol (CL), ammonium formate, sodium chloride, hydrochloric acid, potassium hydroxide, naproxen, indomethacin, phenytoin, fenofibrate, griseofulvin, carvedilol and tadalafil were purchased from Merck Chemicals Ltd. Lecithin, (phosphatidylcholine from Soybean "98%"), (PL) was purchased from Lipoid company, Germany. Felodipine was purchased from Stratech. Chloroform was purchased from Rathburn Chemical Company. Formic acid, sodium phosphate monobasic monohydrate, 1 mL syringes, 2 mL syringes and 13 mm

Table 1
Final concentration of components in the simulated intestinal fluids

Media	Bile Salt (mM)	Phospholipid (mM)	Free Fatty Acid (mM)	Cholesterol (mM)	pH	[pH x TAC] (mM)
Minimum (1)	1.60	0.17	0.07	0.04	2.41	4.54
Q1 (2)	2.34	0.16	1.18	0.06	7.23	27.04
Median (3)	3.10	0.39	1.69	0.08	7.92	41.63
Q3 (4)	5.43	0.57	2.59	0.12	7.75	67.58
Maximum (5)	36.18	5.78	15.03	0.20	8.01	458.05
Biorelevant (6)	3.00	0.75	-	-	6.50	24.38

membrane, 0.45 μm pore size PTFE syringe-filters were purchased from Fisher Scientific. Acetonitrile was HPLC grade and disposable semi-micro cuvettes were from VWR.

2.2. Methods

2.2.1. Simulated intestinal fluid media preparation

A detailed method for the preparation of the simulated intestinal fluid media can be found in Appendix Table A1 shows the composition of each media point and target pH. The biorelevant FaSSIF media was prepared as per manufacturers guidelines.

2.2.2. Dynamic light scattering (DLS)

Particle size of the samples was measured via dynamic light scattering (DLS) using a Zetasizer Nano-ZS (Malvern Instruments, Malvern, UK). Back-scattered light from a 632.8 nm laser (4 mW output) at an angle of 173 ° at 37 °C was used to determine the particle size distributions and hydrodynamic diameters. Each sample was prepared in the same way as for the solubility measurement then filtered with a 0.45 μm membrane and the initial 0.5 mL of filtrate was discarded prior to remove any particulate matter from the filter; sample was then transferred into a semi-micro disposable cuvette. The instrument was equilibrated for 2 minutes before each measurement and the auto attenuator was used to identify the optimum position for analysis. Three batches were prepared and three measurements were taken for each sample and the mean ± standard deviation for the most prominent peak in the size distribution by intensity from each individual run of 12-16 measurements was reported (n=9). The intensity distribution was chosen as it is the first order result generated by the instrument and does not involve any assumptions regarding the Mie scattering theory, unlike the volume distribution which assumes that the particles are spherical, homogenous and that the optical properties are known. Also assumed is that the intensity distribution is correct (Dynamic Light Scattering - Common Terms Defined, xxxx). The number of measurements taken was high to account for the polydispersity of the sample.

2.2.3. Nanoparticle tracking analysis (NTA)

Nanoparticle tracking analysis measurements were performed with a NanoSight instrument (Malvern, UK), both the NanoSight NS300 and NanoSight Pro systems were used, depending upon instrument availability, with a 488 nm blue laser. The NS Pro instrument was used to analyse most of the samples, while the NS300 instrument was used to measure all samples of the maximum media point. The NS300 was also used to record data on the fresh blank Q3 sample, as well as the drugs griseofulvin, carvedilol and tadalafil in the Q3 media SIF.

All samples were diluted with ultrapure Milli-Q water, using Gilson pipettes (P10, P200, P1000, P5000) to a suitable dilution to enable analysis to occur. The dilution factor was of a range of 10-2000, this varied depending on drug and media as per manufacturer guidance, optimum measurement for this instrument is 20-80 particles per frame with the measured concentration range is 10^6 to 10^9 particles per mL.

The drug loaded samples in the new suite of media were buffered with sodium phosphate monobasic monohydrate while the biorelevant samples contain monobasic sodium phosphate dihydrate buffer. The fresh blank and blank 24 hours samples in the new suite of media do not contain buffer.

Triplicate measurements on each sample were taken consecutively at 37 °C, each consisting of 5 captures with 750 frames per capture (each capture was 1 minute in duration), using the NTA instrument script capability. The syringe pump speed for analysis was 1.5 mL/minute and the distribution type used was “raw” which is recommended for polydisperse samples. The instrument was flushed with deionised water between samples. After video capture, the next stage of analysis was data processing of the raw data.

2.2.4. Data Processing

Data processing was carried out using the instrument software, NS Xplorer version 1.0 (Pro) NS XPLORER - v1.0.8641.14 and NanoSight NTA software version 3.4 (NS300). The NS Xplorer software automatically processes the data collected in the background, producing the record information and data graphs of size (nm) vs. concentration (particles/mL). The detection threshold, which sets the minimum brightness of pixels considered for tracking, is manually set when using the NS300 instrument. If this is set too low, more pixels that may be particles are detected while at the same time background noise can be tracked. On the other hand, if this is set too high, pixels that are particles will be excluded from the data collection. Once processed, the data can be exported for further analysis. The modal particle size is generally used to describe the particle size rather than the mean, due to the non-parametric/skewed nature of the data sets.

2.2.5. Data analysis

Data analysis and comparison was conducted using GraphPad Prism 10.1.2. and OriginPro 2022. The solubility and particle size data are compared to values from literature that have used the same drugs in versions of FaSSIF (Klumpp et al., 2019; Teleki et al., 2020; Augustijns et al., 2014; Kloefer et al., 2010). The Mann-Whitney test was performed to identify the statistical significance between the particle size data measured by DLS compared to that measured by NTA.

2.2.6. Equilibrium solubility measurement

Solubility studies were conducted in triplicate and using the method previously reported (Abuhassan et al., 2021). This protocol has been previously validated to ensure equilibrium solubility is achieved post 24 hours with no methodological interference (Khadra et al., 2015; Perrier et al., 2018; Zhou et al., 2017). An excess of drug above its solubility limit (approximately 14 mg) was added to 15 mL centrifuge tubes (Corning® tubes). Equal aliquots (each of 267 μL) of simulated intestinal media (as described in Table 1), buffer (sodium phosphate monobasic monohydrate, 28.4 mM), salt (sodium chloride, 105.9 mM) were then added to the tubes and water (3.199 mL) was added to complete the final aqueous system to a total volume of 4 mL. The pH was adjusted to target value ± 0.02 using KOH and/or HCl as required (no more than 10 % of the final volume was added during pH adjustment). The tubes were placed on an orbital shaker for 1 hour after which the pH was adjusted if required according to the target values in Table 1. Tubes were secured in a rotary shaker at 37 °C for 24 hours. Post-incubation, 1 mL from each tube was centrifuged in a 1.5 mL centrifuge tube (Eppendorf® tube) for 15 minutes at 10,000 rpm then the supernatant was analysed by HPLC.

2.2.7. HPLC analysis

HPLC analysis was performed on a Shimadzu LC-2040C 3D HPLC instrument using a gradient method. The column used was Xbridge® C18 5 µm (2.1 x 50 mm) at 30 °C. Mobile phase A was made by adding 1576.5 mg (10 mM) of ammonium formate to 2.5 L of deionised water. This was adjusted to pH 3.00 (± 0.1) with formic acid. Mobile phase B was made by adding 1576.5 mg (10 mM) of ammonium formate to HPLC grade acetonitrile:water (9:1), total volume of 2.5 L. Flow rate used was 1 mL/min (with the exception of carvedilol where 0.7 mL/min was used). The gradient used for analysis can be found in Table 2.

The full run for each sample was a total of 8 min. The retention time, analysis wavelength, injection volume and flow rate for each active pharmaceutical ingredient is found in Table 3.

3. Results and Discussion

3.1. Dynamic Light Scattering

The results of the particle size analysis by DLS can be found in Figure 1 and the raw data in Appendix Table A2. The samples designated "Fresh Blank" and "Blank 24 hours" are both drug-free media that is analysed fresh (approximately 2 hours postproduction) and 24 hours postproduction, respectively. DLS measurements of blank buffer (no bile/phospholipids etc.) were attempted although it was not found to be possible to determine any size data. This indicates, as anticipated, that there are no particles present.

Figure 1 shows the size and intensity distribution measured for the SIF media. (The size and intensity distribution presented by drug can be found in Appendix Figure A1). There is no variation in particle size between the fresh and 24 hour blank Minimum media, however a noticeable difference can be seen for the Q1 media where the distribution measured in the blank Q1 media point has become multimodal at the 24 hour time point, with small peaks appearing around 5 and 10 nm. The z-average recorded for fresh blank Q1 media was 59 ± 7 nm (compared to the primary peak intensity distribution of 100 ± 2 nm) and the PDI was 0.5 ± 0.1, after 24 hours the z-average decreased to 44 ± 19 nm while the PDI increased to 0.8 ± 0.2. In essence, the average size across the distribution decreases while the polydispersity of the blank media increases to broad size distribution as time is increased.

The concentration of sodium taurocholate is 1.60 and 2.34 mM in the Minimum and Q1 media points, which is lower than the ~3 mM CMC typically reported for this bile salt (Parekh et al., 2023; Clulow et al., 2017; McGown and Nithipatikom, 1988). However, as this data shows colloidal structures are detected in both media. It is also possible for the other components to associate with the bile salt to form colloidal structures. A comparable theory is the formation of mixed micelle structures from both bile salts and phospholipids. It has previously been shown that this could create a mixed bilayer disk structure with bile salt molecules surrounding the boundary of the disk which would result in a diverse range of structure sizes leading to polydispersity of the sample (Mazer et al., 1980).

The size of the structures in the minimum fluid are small yet they increase by at least 10-fold from the blank media at the 24 hour time-point. There are further increases in the presence of drug compared to the fresh media ($p<0.05$),which may indicate that the drug is interacting with the colloidal structures perhaps stabilising the colloids into some larger aggregate. In the Q1 media the size of the blank and drug containing colloidal structures exhibit the opposite behaviour to the minimum where larger colloidal structures are measured in the blank media which decrease in size by around 6-fold, from a particle size of >100 nm to around 15-30 nm in the presence of drug (with the exception of carvedilol).

Polydispersity was observed for the blank Q1 media after 24 hours where some of the smaller particles only present at 24 hours are the same size as those present in the blank minimum media (both fresh and at 24 hours). The drug containing colloids seem to be smaller for each drug in the Q1 media compared to the minimum which is interesting as this opposes the trend seen for the blank media.

The pattern of peaks for the median fluid is similar to that observed for Q1 where there was a change from fresh to 24 hour blank media of an increase in the proportion of smaller particles. Polydispersity was observed for the same drugs in the Median media as for those with Q1 media: felodipine; griseofulvin and tadalafil.

The Q3 media shows much more polydispersity compared to that observed for the minimum; Q1 and median fluids. As with all previous media after 24 hours there was a significant proportion of colloidal structures <10 nm in diameter in the blank media. With the exception of naproxen, all drug containing media show multi-modal distribution of colloidal particles. In many cases there are two distinct populations; those with a size of around 100 nm and those about 10 nm. It would be very interesting to know more about the composition of these structures. The change in Q3 compared to the previous media discussed (minimum; Q1 and median) is the appearance of a colloidal structure at 100 nm. It may be that the increased [TAC] enables these structures to be formed whereas the previously lower concentrations meant that these structures were not present.

A large decrease in particle size measured in the maximum media point to around 5-6 nm. The structures that were present for the Median and Q3 media at 100nm are no longer present at the same intensity for all drugs.

The biorelevant media shows a more consistent size of ~100 nm with no change from fresh to the 24 hour sample. This is similar to the profiles observed for the median and Q3 media, however, it is a quite different profile to the other fluids which may have implications of the structures likely to be formed in vivo and how this may affect the overall solubility.

All measurements were recorded at 37 °C. Comparing this to size measurements by Kloefer *et al.* who analysed blank FaSSIF media using DLS (Zetasizer ZS, Malvern Instruments), measured the colloidal particle size to be 49.2 ± 0.4 nm. However, Kloefer *et al.* used the z-average and polydispersity index to collect their results which uses a cumulant method (Kloefer et al., 2010) while the data in this study was collected using the mean value of the primary peak of the intensity distribution. The z-average recorded for Fresh Blank Biorelevant FaSSIF was 77 ± 1 nm while the polydispersity index (PDI) was found to be 0.03 ± 0.01. As the z-average is very similar to the primary peak intensity recorded for the Fresh Blank Biorelevant media (81 ± 2), this indicates that the fresh blank biorelevant FaSSIF media is monodisperse. After 24 hours, the blank Biorelevant FaSSIF media had a z-average of 67 ± 2 nm and a PDI of 0.03 ± 0.02, compared to the primary peak intensity measured of 70 ± 3 nm. Guidance states that samples with a PDI smaller than 0.05 are

Table 2
Gradient method used for HPLC analysis

Time (min)	Mobile Phase A (%)	Mobile Phase B (%)
0.0	70	30
3.0	0	100
4.5	70	30
8.0	0	0

Table 3
HPLC conditions for each drug material

Drug	Retention time (min)	Detection (nm)	Injection volume (µL)	Flow rate (mL/min)
Naproxen	1.3	254	10	1.0
Indomethacin	1.8	254	10	1.0
Phenytoin	1.1	254	20	1.0
Felodipine	2.4	254	10	1.0
Fenofibrate	2.7	291	10	1.0
Griseofulvin	1.2	291	10	1.0
Carvedilol	1.3	254	10	0.7
Tadalafil	1.4	291	50	1.0

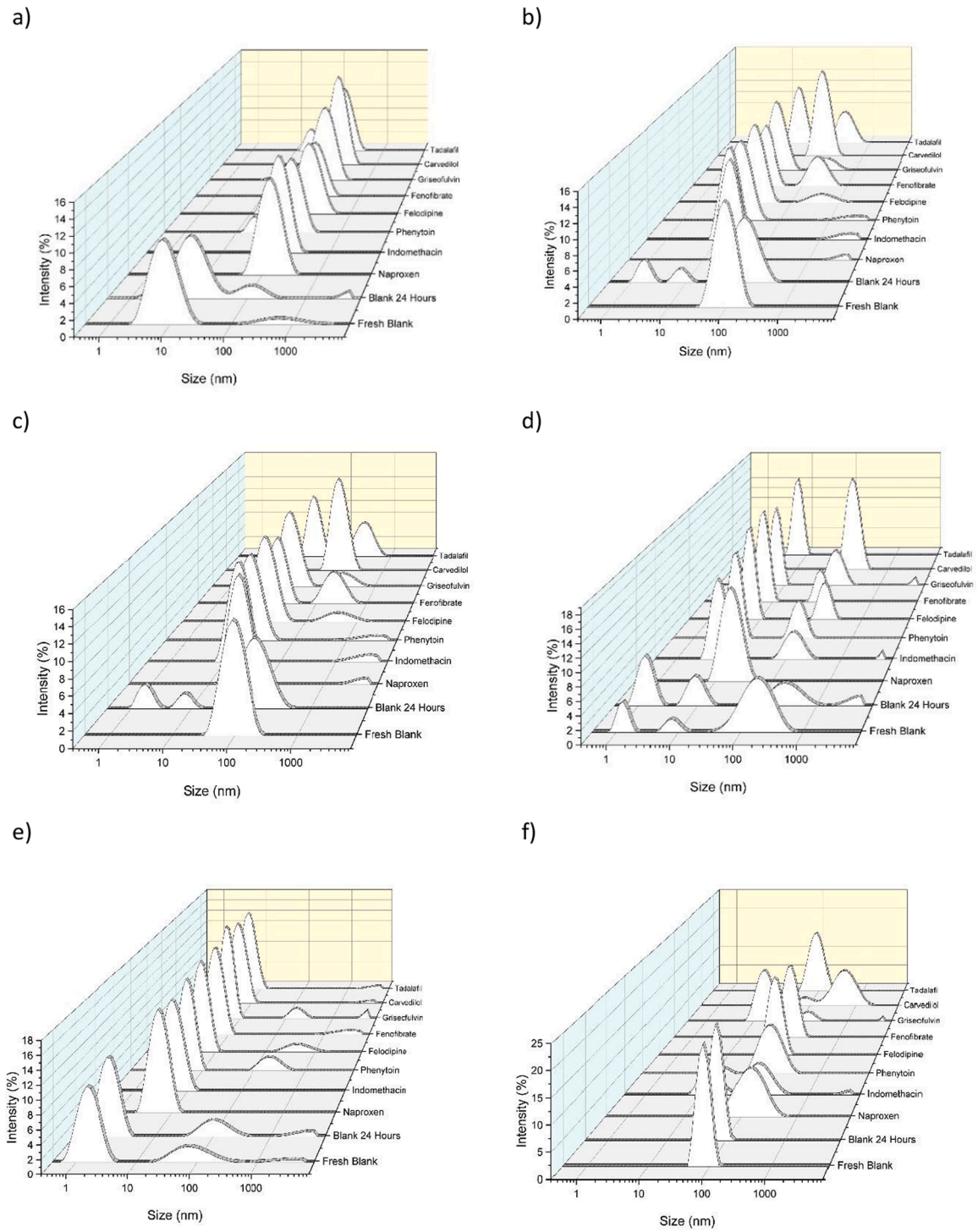

Figure 1. DLS size and intensity distribution for the a) Minimum b) Q1, c) Median, d) Q3, e) Maximum and f) Biorelevant FaSSIF V1 media

infrequently observed other than with highly monodisperse standards while samples with a PDI greater than 0.7 indicate a very broad size distribution (Dynamic Light Scattering - Common Terms Defined, xxxx). The z-average recorded for the fresh blank minimum media was 7.5 ± 0.3 nm and the PDI was 0.25 ± 0.01 which increased after 24 hours to 16.4 ± 5.3 nm and 0.44 ± 0.09, respectively. This emphasises that this suite of SIF media is fairly polydisperse therefore the intensity data was used for data analysis.

Prior to DLS analysis, the samples were filtered through a 0.45 μm filter. However, from the intensity distribution of some of the drugs it can be seen that the instrument has detected particles greater than this size. It may be possible that the colloidal units formed are flexible and are able to navigate through the membrane. A different theory is that the structures split apart, then once through the membrane they then coalesce into the larger arrangements, or as there are also some larger particles in the blank media they may simply be foreign particles.

Although this study is focused on the fasted state with the media points created from real data from a clinical study analysing fasted state human intestinal fluid, the composition of the maximum media point is closer to that of the fed state. Biorelevant FeSSIF is created with a bile salt concentration of 15 mM and a phospholipid concentration of 3.75 mM^3, generating a bile/lecithin ratio of 4:1. Comparing this to the

composition of the maximum media point which has a concentration of 36.18 mM and 5.78 mM for bile salt and phospholipid, respectively giving a ratio of approximately 6.26:1. Xie *et al.* analysed the micelles formed in biorelevant FaSSIF v1 and FeSSIF by DLS and the results of this study characterised micelles of a mean size of 7 nm in the fed state and 78 nm in the fasted state which is in agreement with the data measured by DLS in this study (Xie et al., 2014), as the fresh biorelevant FaSSIF media was recorded to have a hydrodynamic diameter of 81 ± 2 nm. Xie et al did not report details on the method used to report size and it has been assumed that the z-average was used. In parallel to this difference in size distribution there is also typically an increase in solubility in the fed state which may be related to the density of colloidal structures or possibly the increase in surface area whereby the smaller size increases the overall potential for drugs to interact with these colloidal structures from within the media.

3.2. Correlation of colloidal size and solubility data

The correlation between solubility and particle size data measured by DLS for each of the drugs in the SIF media is shown in Figure 2. A similar trend is observed for all three acidic drugs, where the drug in the minimum and biorelevant media points have a lower solubility and greater particle size (between 100-200 nm) than the other four fluids which measure a much greater solubility but lower particle size (<50 nm). It is interesting that the plots of indomethacin and phenytoin show the data points for the different media in the same location relative to the others e.g. the maximum media point has the greatest solubility and lowest particle size which is of a slightly higher solubility than that of the drug in the Q3 and median fluid points, which are of a similar solubility but the particle size is measured to be slightly larger in the median SIF than the Q3 media. As the concentration of amphiphiles increases with increasing media point, the solubility of drug also increases and with that there is a decrease in particle size of the colloidal structures formed. This may be due to lipophilic drugs being solubilised into the hydrophobic core of the micelle structure and increased interactions between drug and micelle.

A similar trend is found in the neutral drugs in the SIF media as observed in the acidic drugs. As expected, the minimum point shows the lowest solubility values with the greatest particle size recorded and the highest solubility and smallest particle sizes were measured in the maximum SIF media points. The solubility of the drugs in the Biorelevant (FaSSIF V1) media is similar to that of the Q1, median and Q3 fluids, however, for felodipine and fenofibrate the particle size in the Biorelevant media is closer to that of the minimum media. This suggests that the drug is solubilising to the same extent as within the SIF media but there are potentially interactions between felodipine/fenofibrate and the composition of the Biorelevant media that results in a larger particle size measurement. This is not observed in griseofulvin so it is not strictly a phenomenon associated with neutral drugs.

Observation of the data from the basic drug carvedilol indicates that the relationship between solubility and particle size is dissimilar to the other acidic and neutral drugs analysed. For this drug larger colloidal structures were measured, with the mean particle size measured in the minimum/Q1/median/Q3 media all >121 nm. In addition, a very high solubility was recorded for the minimum media which was not observed for other drugs. Carvedilol also showed an unusually large particle size in the Biorelevant FaSSIF media that was greater than the filter pore size for these solutions which indicates coalescence of the colloidal structures.

Tadalafil follows the same trends as the other basic drugs although the solubility is much lower while the particle size is similar to the acids and neutrals. Interestingly, the solubility data for tadalafil is very similar to that observed for fenofibrate, although the chemical structures and physicochemical properties are not particularly similar. The general trend recorded in nearly all of the drugs indicates that high solubility is linked to small colloidal particle size.

There is a considerable spread of lipophilicity of the drugs analysed in this study, ranging from the antifungal drug griseofulvin with a logP value of 2.18, to the antilipemic drug fenofibrate with a logP of 5.2[7]. All drugs analysed possess a positive logP value, indicating that the drugs are more lipophilic than hydrophilic. Naproxen and indomethacin have pKa values of 4.15 and 4.5 therefore they will be nearly fully ionised in all media with the exception of the minimum media. When in the ionised form, it is anticipated that they will associate with the hydrophilic outer colloidal layer which may influence the size of the resulting colloidal structure. Carvedilol has a pKa of 7.8, therefore the majority of the drug will be in an ionised form in all media other than the median and maximum which have pH values of 7.92 and 8.01. It is expected that it will be nearly fully ionised in the minimum media where the pH is 2.41 and less ionised in the other media in which the pH is greater at around 7 pH units. The weakly basic drug tadalafil has a low pKa value at 3.5 which indicates that the majority of this drug will be in the non-ionised (protonated) form in the acidic pH of the minimum media, while it will be in the ionised form in the other SIF media where the pH is greater than the pKa. The calculated degree of ionisation for the acidic and basic drugs analysed can be found in Table 4. The pKa values have been sourced from literature.

3.3. Nanoparticle tracking analysis

NTA was used to measure the concentrations and modal size distributions of the samples, which could then be compared to the measurements recorded by DLS. The mean and standard deviation of the modal distributions measured by NTA can be found in Appendix Table A3. Many of the samples were measured to have a particle modal size of between 60 and 80 nm.

The Fresh Blank Biorelevant media was measured to have a modal size distribution of 61 ± 3 nm which typically did not vary considerably after 24 hours or on the addition of drugs. A larger particle size by carvedilol is recorded both by NTA where the modal size is measured to be 114 ± 6 nm and by DLS where the primary peak is measured to occur at 624 ± 13 nm. A Mann-Whitney test was conducted and a significant difference ($p<0.05$) was found between the particle size measurements for every drug in every media recorded by DLS compared to that recorded by NTA. The results of this test can be found in Appendix Table A4. Interestingly, in both measurements, the structures of carvedilol in the biorelevant media are the greatest measured of any drug in the biorelevant media. This suggests that carvedilol is interacting with the multimolecular aggregates resulting in the formation of larger structures.

The largest discrepancy between the two techniques lies within the Q3 and maximum media points. The DLS data measured drug loaded structures of sizes around 5 and 6 nm while for the same drugs and media measured by NTA, much larger sizes were recorded. As the drug loaded Q3 and maximum samples are diluted, this dilution probably explains the lack of very small particles observed, as previously size decreased with increasing [TAC]. A still image taken from a video recording of phenytoin in the minimum SIF media is shown in Figure 3. The video recording can be found in the Supplementary Information.

It is necessary to dilute samples before analysis by NTA. To identify optimum sample concentration for analysis, sequential dilutions were made and the final dilutions of each sample prior to analysis is shown in Table 5. The samples were diluted systematically to a factor of 1000 and the results indicated that the micelles above the CMC are reduced to their fundamental constituent molecules once the dilution is below the concentration at which molecular aggregation occurs (Polat et al., 2020). Despite the dilutions that may take the sample concentrations below the CMC, particles were observed and sizes were measured for these dilute systems. The results of which are shown in Figure 4.

The modal size distributions of the SIF media measured by NTA, with and without drugs can be found in Figure 4. Most of the sizes determined lie between 50 to 100 nm with a few outliers. Typically, the drug

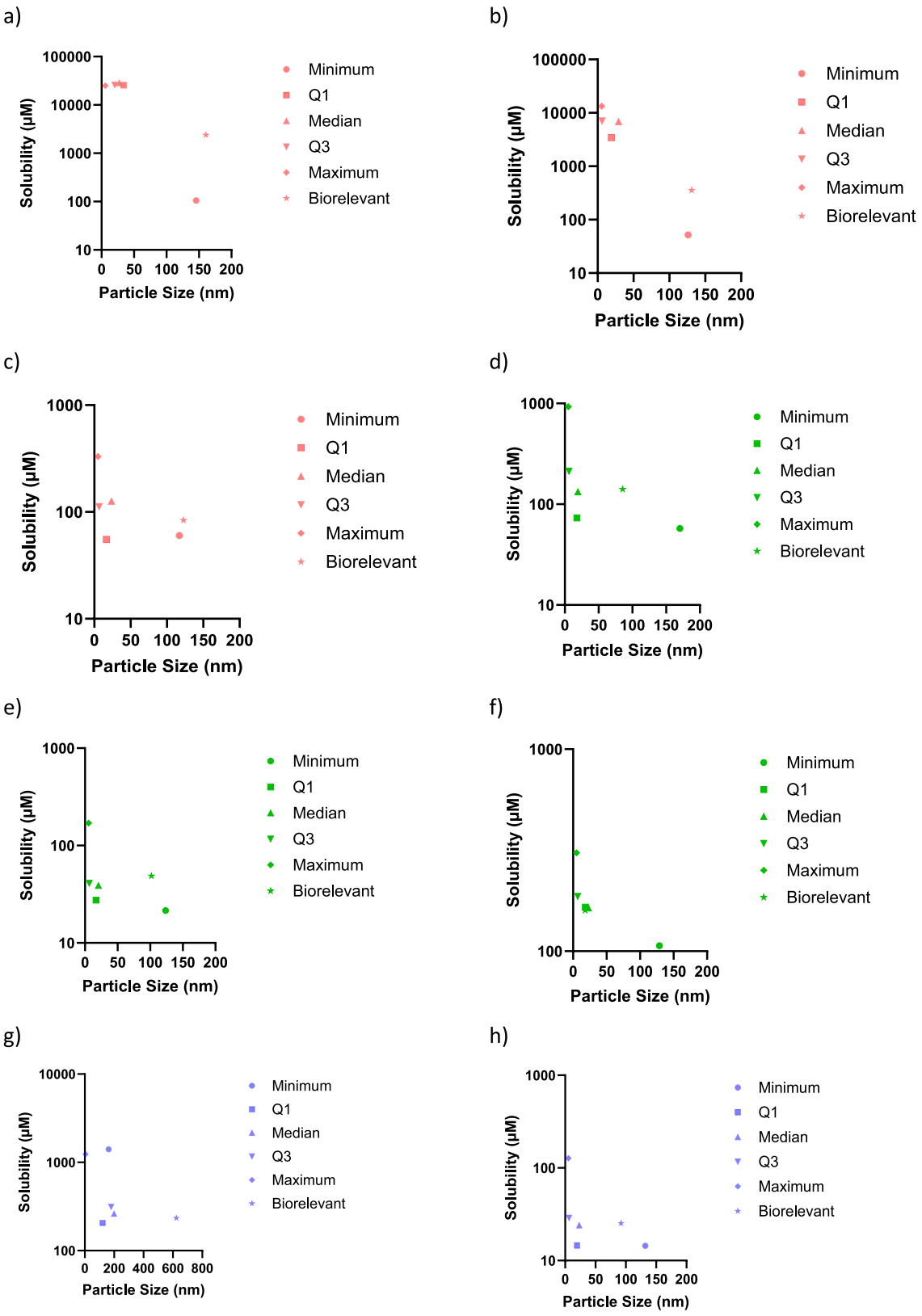

Figure 2. Plot of solubility and particle size measured by dynamic light scattering of a) naproxen b) indomethacin c) phenytoin d) felodipine e) fenofibrate f) griseofulvin g) carvedilol h) tadalafil in each of the SIF media. Note that the y-axis scales differ for each drug for clarity of data presentation.

Table 4
Degree of drug ionisation of acidic and basic drugs analysed, calculated from reported pKa values and pH of SIF media

pKa	Drug	Media	Minimum	Q1	Median	Q3	Maximum	Biorelevant
		Media pH	2.41	7.23	7.92	7.75	8.01	6.50
4.15	Naproxen		2.2 %	99.9 %	99.9 %	99.9 %	99.9 %	99.6 %
4.5	Indomethacin		0.9 %	99.8 %	99.9 %	99.9 %	99.9 %	99.0 %
8.33	Phenytoin		99.9 %	92.7 %	72.1 %	78.9 %	67.2 %	98.5 %
7.8	Carvedilol		99.9 %	78.8 %	43.1 %	52.9 %	38.1 %	95.2 %
3.5	Tadalafil		7.9 %	99.9 %	99.9 %	99.9 %	99.9 %	99.9 %

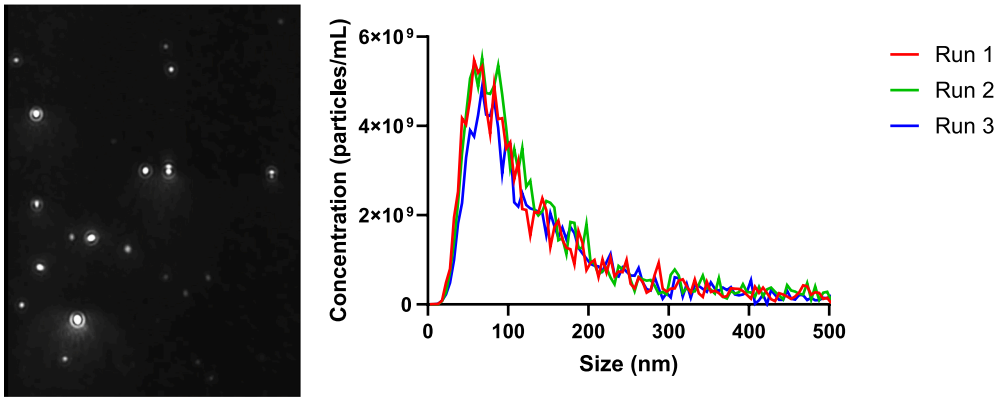

Figure 3. Video frame captured by the NanoSight Pro instrument showing light scatter from polydisperse phenytoin mixed micelle structures in the Minimum SIF media. Modal size distribution measured for this sample was 64.17 ± 5.77 nm with corresponding size distribution graph.

Table 5
Sample dilutions prior to analysis by NTA

Drug	Media					
	Minimum	Q1	Median	Q3	Maximum	Biorelevant
Fresh Blank	1000	1000	1000	50	500	5000
Blank 24 hours	1000	1000	1000	1000	1000	5000
Naproxen	1000	1000	1000	1000	100	2000
Indomethacin	1000	10	1000	100	10	1000
Phenytoin	500	1000	1000	1000	10	1000
Felodipine	1000	100	1000	1000	100	5000
Fenofibrate	500	100	500	1000	100	5000
Griseofulvin	500	1000	500	100	50	1000
Carvedilol	1000	1000	1000	100	50	500
Tadalafil	1000	1000	1000	100	50	1000

solubilised in the maximum media point results in the greatest modal size measured with a few exceptions. Fenofibrate, where the largest size measured is in the Q1 media, although there is a large standard deviation recorded for the maximum media point. The largest particle size for griseofulvin was also measured to be in the Q1 media point. For carvedilol, the largest sized particles were found to be in the biorelevant media, although taking standard deviations of the samples into account, the particles are not significantly different in size to those in the minimum or Q1 media. This does not agree with the data recorded by DLS, where the greatest mean particle size measured with either in the minimum media point, or in the biorelevant media point. It is likely that the dilution of the samples alters the colloidal structures formed, especially in the higher [TAC] media points. As discussed previously, where the [TAC] is greatest in the maximum media point, this SIF sample is closer to that of the fed state and the addition of the diluent at such high volumes may alter the colloidal structure of the drug loaded micelles resulting in structures that are a ten-fold increased in size than those detected by DLS.

The solubility and particle size data measured by NTA for each of the drugs in the SIF media is shown in Figure 4.

In contrast to the DLS data the NTA typically showed that solubility increased with particle size of colloidal material present. These data are affected by the dilutions required for measurements yet it was interesting to observe the structures formed and the concentration of these structures that was possible to measure via NTA whilst not possible with DLS. NTA data is limited due to this requirement for dilution. In addition, a cross validation of the results obtained by NTA using the different instruments would be valuable for future data if NTA is to be explored further to ensure that the two instruments provide confidence that the data from these two instruments are comparable. For the purposes of this study the data presented demonstrates that measurements via NTA is possible, yet some methos optimisation is required.

Table 6 summarises and highlights the variation in measured diameter of FaSSIF in previous literature and compares the values to those measured within this paper. Klumpp *et al.* measured the particle size of FaSSIF V1, V2 and V3 using DLS (Zetasizer ZS, Malvern Instruments) and recorded the size of the media particles to be 64.31 ± 6.27, 29.31 ± 2.47 and 11.13 ± 1.12 nm respectively, 2 hours postproduction at room temperature. Particle geometry analysis by cryogenic transmission electron microscopy detected various structures within the media including vesicles, disc micelles (singular and agglomerates), thread-like micelles and globular micelles which further demonstrates the

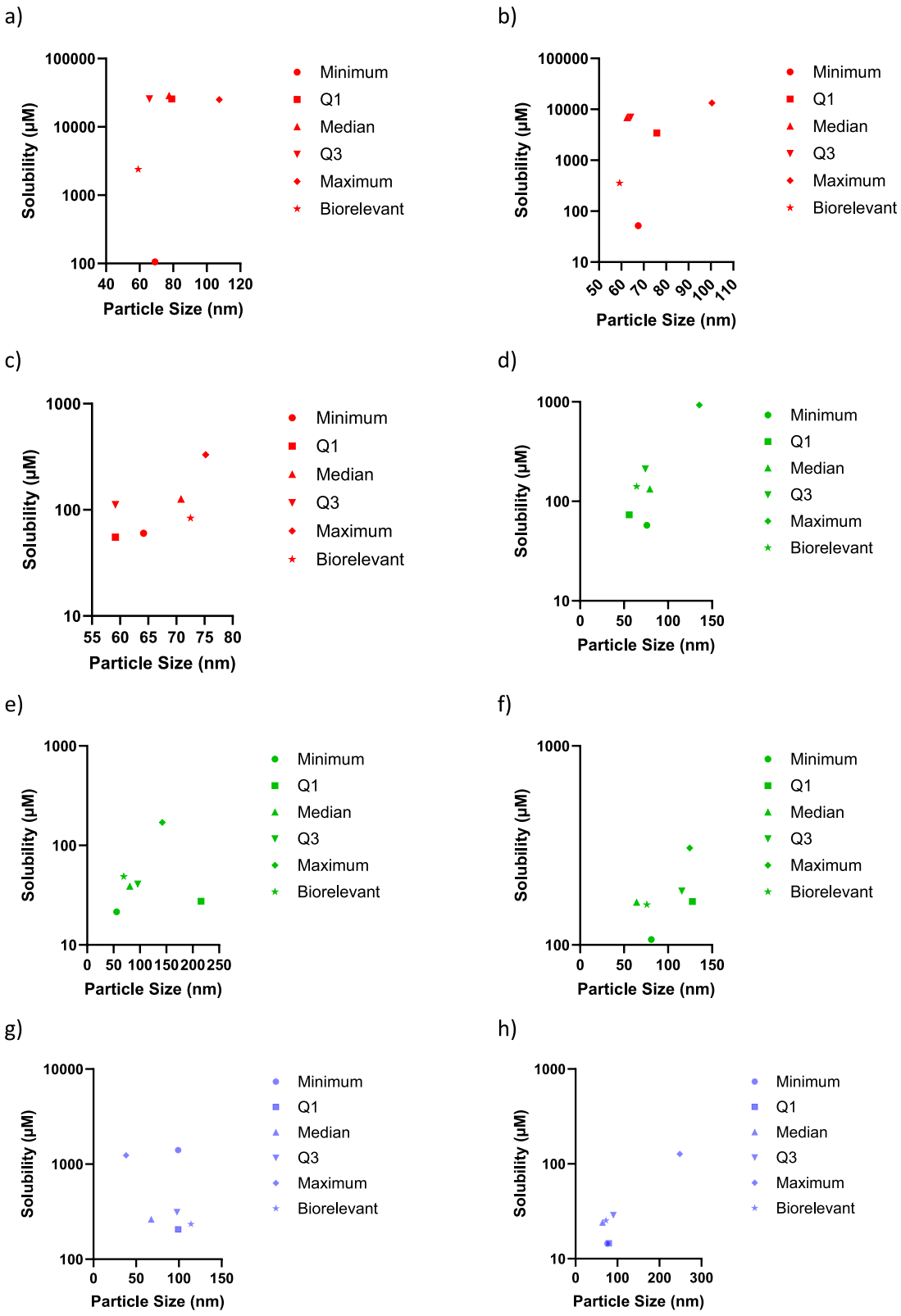

Figure 4. Plot of equilibrium solubility of the drug in the undiluted media and particle size measured by nanoparticle tracking analysis of a) naproxen b) indomethacin c) phenytoin d) felodipine e) fenofibrate f) griseofulvin g) carvedilol h) tadalafil in each of the SIF media. Note that the axis are scaled for each plot according to the data presented for clarity.

Table 6
Particle diameter of various FaSSIF media measured 2 hours postproduction.

FaSSIF Media	Particle Diameter (nm)	Source
Biorelevant FaSSIF V1	81 ± 2	This study (DLS)
Biorelevant FaSSIF V1	61 ± 3	This study (NTA)
Biorelevant FaSSIF V1	49.2 ± 0.4	Kloefer *et al.* (Kloefer et al., 2010) (DLS)
Biorelevant FaSSIF V1	48 ± 6.6	Clulow *et al.* (Clulow et al., 2017) (DLS)
Biorelevant FaSSIF (FaSSIF V1)	64.31 ± 6.27	Klumpp *et al.* (Klumpp et al., 2019) (DLS)
FaSSIF V2	29.31 ± 2.47	Klumpp *et al.* (Klumpp et al., 2019) (DLS)
FaSSIF V3	11.13 ± 1.12	Klumpp *et al.* (Klumpp et al., 2019) (DLS)
FaSSIF mixed micelles	91 ± 10	Lehto *et al.* (Lehto et al., 2011) (DLS)

polydispersity of the media (Klumpp et al., 2019). Despite the dilution of the Biorelevant FaSSIF in this study the NTA data shows a similar particle size to these other data sets which provides some reassurance of the value of this method for measurement of colloidal structures in simulated intestinal fluids.

Furthermore, Clulow *et al.* measured the particle size of blank FaSSIF V1 (Biorelevant.com) by DLS, using the intensity distribution by calculating the mean of the primary peak, to be 48 ± 6.6 nm. The wide range in particle size of the media reported could potentially be caused by the composition of media. Here, the media was made using sodium taurocholate, lecithin, sodium oleate, cholesterol, buffer and salt, while in the other studies the authors used Biorelevant FaSSIF powder and buffer concentrate. Lehto *et al.* measured the sodium taurocholate/lecithin FaSSIF mixed micelles to have a hydrodynamic micellar radii of 91 ± 10 nm which is considerably closer to the data measured in the minimum and Q1 media points (Lehto et al., 2011). After NTA analysis, where the samples have been diluted with water for optimum particle analysis concentration, the particle size measured for the fresh Biorelevant FaSSIF was 61 ± 3 nm. This suggests that colloidal structures are still present, even after the dilution required the analysis.

Table 6 highlights the large differenced reported in the size of colloidal structures in FaSSIF media. The study by Klumpp *et al.* clearly shows that the composition of FaSSIF influences the size of the colloidal structures formed. The other studies show that there are inter-laboratory differences in measured size that may be related to processing differences for the same composition or that subtle changes in composition affect the size. For example, there may be batch to batch variation in the components of FaSSIF between laboratories that affect sizes formed. As discussed previously there are also differenced in whether it is the z-average or intensity data that is reported from the DLS analysis.

3.4. Solubility

The mean equilibrium drug solubility results, where n=3, of the eight drugs analysed in the six fasted state media points are shown in Figure 2. The raw data can be found in Appendix Table A5.

The solubility of weakly basic biopharmaceutical classification systems (BCS) class II drugs such as carvedilol and tadalafil are pH dependent. The solubility of these two drugs in the suite of media studied ranged from 1406 ± 34 to 1235 ± 27 μM (carvedilol) and 14.4 ± 0.5 increasing to 127.3 ± 2.1 μM (tadalafil).

A large increase in solubility is observed between the Q3 and Maximum media point for carvedilol (313.7 ± 2.6 μM increasing to 1235.5 ± 27.1 μM). This increase is interesting as the pH of the maximum media point is now greater than the pKa value of the drug (7.8 (Berkhout et al., 2021) which is contrary to the expected behaviour where the solubility of the weakly basic drug should decrease when pH > pKa. This is also observed in tadalafil between these two SIF points, but is unrelated to pKa as the pH of the media does not exceed the pKa of tadalafil (pKa = 10^6). The data show that the solubility is linked to the [TAC] to a greater extent than the pH for these weak bases. The equilibrium solubility of carvedilol in the minimum media is measured to be 1406 ± 34 μM while the pH of the media is 2.41. Carvedilol will be in the fully ionised form and displays the standard pH-dependent solubility profile at low pH of a weakly basic drug where a high solubility is observed. This data is in agreement with work carried out by Hamed *et al.* that investigated the solubility and dissolution behaviour of carvedilol in various simulated gastric and intestinal fluids. The researchers recorded a saturation solubility of carvedilol in blank simulated fasted state gastric fluid at a low pH of 1.6 to be 2399 ± 41 μg/mL (5900 ± 100 μM) (Hamed et al., 2016).

The neutral drugs analysed (felodipine, fenofibrate and griseofulvin) have similar solubility values within the suite of media, which steadily increase with increasing [pH x TAC] in the media. From media point 1 to media point 5, there appears to be a linear increase of in solubility in parallel to the increase in [pH × TAC]. There is a considerable increase in all three drug solubilities between media Q3 and the Maximum media point. This is related to the non-linear increase in [TAC] in these media.

As expected, the acidic drugs analysed (naproxen, indomethacin and phenytoin) have a higher solubility value in the intestinal fluid, compared to the neutral and basic drugs. As the drugs are weakly acidic, they have a considerably lower solubility in the minimum media point where the pKa values are greater than that of the pH (2.41 ± 0.02) at 4.15, 4.5 and 8.33 respectively (Wishart et al., 2006). As the pH of the media increases to the Q1 media point to a value of 7.23 ± 0.02 there is a substantial increase in solubility of the naproxen and indomethacin which is a result of the pH being greater than that of the pKa values. There is a slight increase at the median media point due to a higher pH value than the Q3 point (7.92 ± 0.02 and 7.75 ± 0.02, respectively). A large increase in solubility should be observed for phenytoin if it were added to a media with a pH greater than 8.33, although that is beyond the scope of this work as the pH of our sample data does not reach this.

4. Conclusion

This study used a new suite of simulated intestinal fluid created encompassing the composition variability revealed from a range of human intestinal fluid samples from a clinical study. The solubility of eight poorly soluble drugs and particle size of the colloidal structures within the SIF was analysed by high performance liquid chromatography, dynamic light scattering and for the first time, nanoparticle tracking analysis.

It was found that the solubility measured was typically greater in the acidic drugs than in the neutrals or bases and that the solubility tended to increase with increasing media point ([pH x TAC]). The particle size of the colloidal structures formed measured by DLS showed a decrease in particle size with increasing amphiphile concentration, both the primary peak intensity and the full intensity distribution were considered due to the polydisperse nature of the structures. Nanoparticle tracking analysis revealed that the majority of samples analysed showed modal colloidal size distributions between 50 and 100 nm although, unlike DLS, the particle size of the structures measured did not fluctuate considerably and the greatest particle size was typically recorded in the maximum ([pH x TAC]) SIF media. DLS data showed that the colloidal structures present changed following the addition of drug compared to drug free media; typically, with a reduction in the size of the colloidal structures formed. The NTA data had increased limitations compared to the DLS data: primarily related to the need to dilute samples prior to analysis but also for this paper the fact that two instruments were used for the data collection.

Further studies using x-ray scattering techniques may provide more information regarding the shape, morphology and orientation of the drug-loaded structures which will enable a deeper understanding of the complex fluid and how the behaviour is affected by the addition of

poorly soluble drugs. The size data recorded in this study can be combined with other solubility data e.g. Khadra *et al.* for further analysis to reveal distinct relationships in this complex fluid (Khadra et al., 2015).

CRediT authorship contribution statement

Zoe McKinnon: Writing – review & editing, Writing – original draft, Methodology, Investigation, Formal analysis. **Ibrahim Khadra:** Supervision. **Gavin W. Halbert:** Writing – review & editing. **Hannah K. Batchelor:** Writing – review & editing, Supervision.

Declaration of competing interest

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.

Data availability

All data available within paper or appendices

Appendix A. . Simulated intestinal fluid media preparation

To create each of the five simulated intestinal media, a concentrated stock solution 15 times the mass was prepared of bile salt (sodium taurocholate), phospholipid (soybean lecithin) and fatty acid (sodium oleate) in chloroform. The mass of each component for the concentrated stock solution can be found in Appendix Table A1. For each of the media recipes, a solution 1500 times greater the mass of cholesterol in chloroform was prepared and an aliquot was transferred to the stock solution as it was not possible to weigh the low amounts of cholesterol required. The chloroform was then evaporated off with a nitrogen gas to produce a dry film which was resuspended in 3 mL of water and stirred to create a homogenous mixture. This was transferred to a 5 mL volumetric flask and made to volume with water. Biorelevant media was prepared as per suppliers' guidelines.

Table A1
Preparation of stock components, mass used to create 15 x stock solution

Media	Bile salt(mg)	Phospholipid(mg)	Free Fatty Acid (mg)	Cholesterol(mg)	pH	[pH x TAC] (mM)
Minimum (1)	64.5	10.0	1.6	116.0	2.41	4.54
Q1 (2)	94.4	9.4	26.9	174.0	7.23	27.04
Median (3)	122.6	23.0	38.6	348.0	7.92	41.63
Q3 (4)	219.0	33.6	59.1	232.0	7.75	67.58
Maximum (5)	1459.0	340.7	343.2	580.0	8.01	458.05

Table A2
Particle Size Analysis by DLS size[a] ± standard deviation (intensity dist. d, nm)

Drug	Media					
	Minimum	Q1	Median	Q3	Maximum	Biorelevant
Fresh Blank	8.51 ± 0.15	100.0 ± 1.6	150.8 ± 5.3	205.2 ± 4.9	1.8 ± 0.1	80.6 ± 1.7
Blank 24 hours	12.5 ± 0.5	132.9 ± 2.3	155.2 ± 6.8	1.4 ± 0.1	1.7 ± 0.1	70.3 ± 2.9
Naproxen	145.7 ± 2.5	34.1 ± 1.4	27.4 ± 1.4	20.3 ± 0.2	5.9 ± 0.4	160.5 ± 3.3
Indomethacin	126.2 ± 2.5	19.1 ± 0.3	29.2 ± 1.6	6.0 ± 0.1	5.7 ± 0.7	131.0 ± 5.8
Phenytoin	117.1 ± 1.5	16.6 ± 0.4	23.5 ± 0.8	6.5 ± 0.1	5.2 ± 0.2	122.9 ± 3.0
Felodipine	170.3 ± 5.4	17.9 ± 0.6	19.3 ± 1.2	6.1 ± 0.1	5.1 ± 0.2	85.6 ± 1.2
Fenofibrate	123.5 ± 3.9	16.9 ± 0.3	20.6 ± 2.5	6.5 ± 0.2	5.5 ± 0.1	101.9 ± 1.9
Griseofulvin	128.9 ± 2.4	18.4 ± 0.2	22.8 ± 1.3	6.8 ± 0.2	5.1 ± 0.3	17.9 ± 1.6
Carvedilol	162.6 ± 2.3	121.8 ± 1.8	199.4 ± 2.2	179.0 ± 3.6	5.3 ± 0.2	623.8 ± 12.9
Tadalafil	132.1 ± 2.3	19.7 ± 0.7	23.0 ± 0.7	6.4 ± 0.1	5.3 ± 0.1	92.2 ± 1.8

[a]Intensity distribution of main peak, average of three runs, three measurements per run on each sample, n=9

Table A3
Particle size analysis by NTA mean of the modal distribution ± standard deviation (nm)

Drug	Media					
	Minimum	Q1	Median	Q3	Maximum	Biorelevant
Fresh Blank	55.8 ± 2.9	75.8 ± 11.5	90.8 ± 2.9	168.6 ± 16.8	175.4 ± 18.3	60.8 ± 2.9
Blank 24 hours	87.5 ± 18.0	89.2 ± 12.6	69.2 ± 7.6	85.8 ± 10.4	106.5 ± 42.8	58.1 ± 1.9
Naproxen	69.2 ± 2.9	79.2 ± 2.9	77.5 ± 5.0	65.8 ± 5.8	107.4 ± 3.5	59.2 ± 2.9
Indomethacin	67.5 ± 0.0	75.8 ± 2.9	62.5 ± 0.0	64.2 ± 2.9	100.4 ± 31.3	59.2 ± 2.9
Phenytoin	64.2 ± 5.8	59.2 ± 2.9	70.8 ± 7.6	59.2 ± 2.9	75.2 ± 2.9	72.5 ± 0.0
Felodipine	75.8 ± 2.9	55.8 ± 2.9	79.2 ± 2.9	74.2 ± 2.9	135.5 ± 6.4	64.2 ± 2.9
Fenofibrate	55.8 ± 2.9	215.8 ± 23.6	80.8 ± 2.9	95.8 ± 5.8	142.3 ± 78.9	69.2 ± 2.9
Griseofulvin	80.8 ± 2.9	127.5 ± 5.0	64.2 ± 5.8	115.6 ± 7.8	124.6 ± 19.4	75.8 ± 2.9
Carvedilol	99.2 ± 2.9	99.2 ± 12.6	67.5 ± 0.0	97.8 ± 2.3	38.1 ± 5.1	114.2 ± 5.8
Tadalafil	75.8 ± 2.9	79.2 ± 5.8	64.2 ± 2.9	90.3 ± 2.2	248.3 ± 49.0	72.5 ± 5.0

Table A4
Result of Mann Whitney test for comparison of DLS and NTA data, P values

Drug/Blank	Minimum	Q1	Median	Q3	Maximum	Biorelevant
Fresh Blank	0.0091	0.0091	0.0091	0.0091	0.0045	0.0091
Blank 24 Hours	0.0091	0.0091	0.0121	0.0091	0.0091	0.0091
Naproxen	0.0091	0.0091	0.0091	0.0091	0.0091	0.0091
Indomethacin	0.0045	0.0091	0.0091	0.0091	0.0091	0.0091
Phenytoin	0.0091	0.0091	0.0091	0.0091	0.0091	0.0061
Felodipine	0.0091	0.0091	0.0091	0.0091	0.0091	0.0091
Fenofibrate	0.0091	0.0091	0.0091	0.0091	0.0091	0.0091
Griseofulvin	0.0091	0.0091	0.0091	0.0091	0.0091	0.0091
Carvedilol	0.0091	0.0091	0.0045	0.0091	0.0091	0.0121
Tadalafil	0.0091	0.0091	0.0091	0.0091	0.0091	0.0091

Table A5
Mean solubility values of drugs analysed in fasted state simulated media ± standard deviation, measured by HPLC (μm).

Drug	Media					
	Minimum	Q1	Median	Q3	Maximum	Biorelevant FaSSIF
Naproxen	105.4 ± 0.4	25633.5 ± 225.1	28609.5 ± 996.2	25736.2 ± 523.2	25009.5 ± 361.2	2391.5 ± 32.2
Indomethacin	51.9 ± 0.1	3433.5 ± 19.5	6842.8 ± 20.7	7045.1 ± 1.5	13310.6 ± 131.9	355.0 ± 4.9
Phenytoin	60.3 ± 1.7	55.3 ± 0.5	125.9 ± 0.9	112.2 ± 1.9	330.1 ± 7.4	83.8 ± 2.2
Felodipine	57.5 ± 0.2	73.2 ± 0.3	132.8 ± 1.5	212.6 ± 2.0	926.6 ± 8.8	140.8 ± 2.7
Fenofibrate	21.5 ± 0.3	27.5 ± 0.2	38.8 ± 0.1	40.9 ± 0.4	170.5 ± 1.3	48.8 ± 0.9
Griseofulvin	106.5 ± 1.8	165.0 ± 4.5	163.7 ± 2.2	186.6 ± 11.7	306.7 ± 6.8	159.4 ± 6.2
Carvedilol	1406.2 ± 33.9	205.9 ± 0.5	261.8 ± 1.4	313.7 ± 2.6	1235.5 ± 27.1	234.1 ± 3.9
Tadalafil	14.4 ± 0.5	14.5 ± 0.1	24.0 ± 0.6	28.9 ± 0.5	127.3 ± 2.1	25.2 ± 0.3

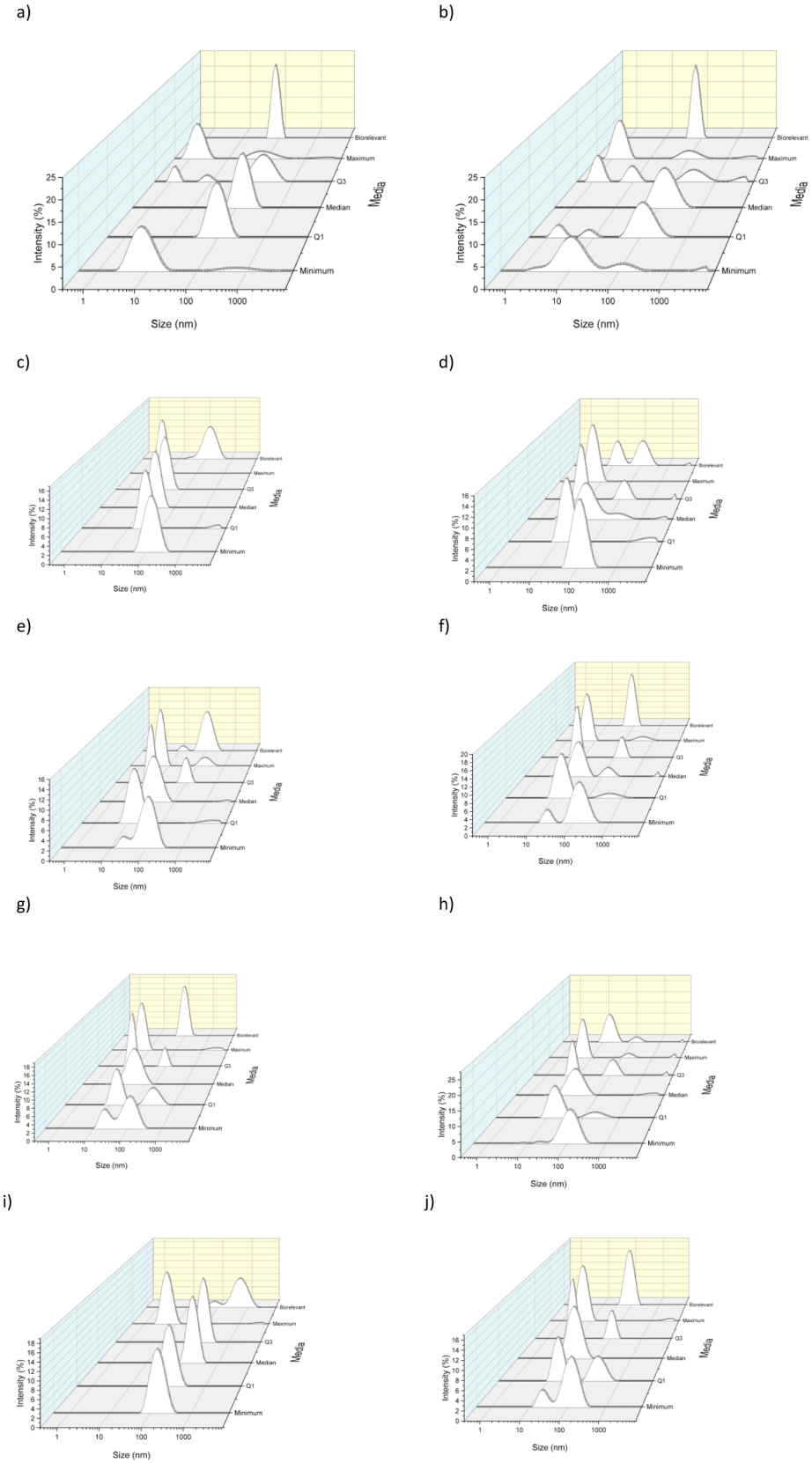

Figure A1. DLS Size and intensity distribution for the a) fresh blank media, b) blank media at 24 hours, c) naproxen, d) indomethacin, e) phenytoin, f) felodipine, g) fenofibrate, h) griseofulvin, i) carvedilol, j) tadalafil

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