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Characterization of the solubility of a poorly soluble hydroxylated metabolite in human urine and its implications for potential renal toxicity

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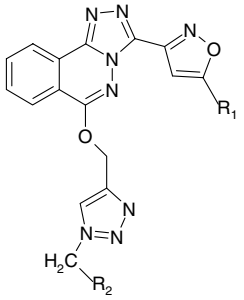
The solubility, in human urine, of the major hydroxylated metabolite (M1) of an experimental cognition enhancer was characterized through a series of *in vitro* experiments in an effort to estimate the probability of crystalluria occurring following oral administration of the parent compound. The aim of these experiments was to determine if a safety margin existed between clinically observed urine concentrations and the solubility of M1. The mean urine concentrations of M1 in young and elderly subjects following oral administration of the parent compound at the highest doses tested, were 4865 ± 2368 ng/mL and 2764 ± 791 ng/mL, respectively. *In vitro* solubility experiments with M1 were conducted in drug-free human urine (37 °C) from four male and four female healthy subjects under conditions of high and low urine osmolality. Mean concentrations ($n = 16$) of M1 in human urine to which solid M1 was added, were 3656 ± 621 ng/mL, 4678 ± 1169 ng/mL and 5378 ± 2474 ng/mL after stirring for 24, 48 and 72 h, respectively, indicating that the *ex vivo* mean solubility of M1 in human urine is no greater than ~5 μg/mL. Addition of solid M1 to urine from human subjects dosed with the parent compound resulted in mean urine M1 concentrations 23.5% greater than those observed *in vivo*.
The results from both experiments indicated a significant overlap between urine concentrations of M1 *in vivo* following the highest oral administration of the parent drug and M1 solubility measured *in vitro*, suggesting a high potential for *in vivo* saturation of urine with M1 with subsequent precipitation, crystalluria, and nephrotoxicity. Consequently, the results of these studies have placed restrictions on the dose that could be administered during clinical development of this compound.

1. Introduction

3-(5-Methylisoxazol-3-yl)-6-(1-methyl-1,2,3-triazol-4-yl)-methyloxy-1,2,4-triazolo[3,4-*a*]phthalazine (compound **1**, Formelschema) is a GABA α-5 inverse agonist that enhances hippocampal-dependent learning and long term potentiation in rodents (Chambers et al. 2002; MacLeod et al. 2003). Compound **1** is metabolized predominantly to a single hydroxylated metabolite, M1. Both compound **1** and M1 have low aqueous solubility (≤0.6 μg/mL) at room temperature and a significant portion of M1 is excreted in urine (compound **1** is not highly excreted in urine).
The low aqueous solubility of M1 raised the possibility that it could precipitate in urine and this was confirmed in animal toxicity studies. The presence of M1 crystals (confirmed by IR and NMR) was observed in rats and monkeys orally administered high doses of compound **1** for up to 14 weeks. Oral dosing of compound **1** in rats resulted in nephrotoxicity that was dependent on both dose and duration of exposure. Female rats dosed at 240 mg/kg/day for 5 weeks developed renal pyelitis and papillitis with crystals present in the kidney lesions. These crystals consisted primarily of the hydroxylated metabolite of com-



Compound 1, $R_1 = CH_3$, $R_2 = H$
Compound M1, $R_1 = CH_2OH$, $R_2 = H$
Assay internal standard, $R_1 = CH_3$, $R_2 = CH_3$

pound **1**, M1. The apparent metabolite dependent nephrotoxicity in the rats suggested the possibility that a similar phenomenon might occur in humans following long-term administration of compound **1**. This hypothesis was based on the fact that significant concentrations of M1 were measured in the human urine collected during clinical trials; the highest concentrations being observed during a two-week multiple dose regimen (8 mg, q8h) in young healthy adults. Experiments were initiated to evaluate the likelihood that precipitation of M1 would occur *in vivo* in humans; these included the determination of solubility of M1 in water, drug-free animal urine, drug-free human urine, and human urine containing M1 from oral dosing of compound **1**.

Several marketed pharmaceuticals have exhibited nephrotoxicity attributable to precipitation of drug and/or metabolites. Examples of such compounds include sulfonamides, methotrexate, acyclovir, and ascorbic acid (Novotny et al. 2000). Solubility-based nephrotoxicity mainly asserts itself in the phenomenon of post-tubular obstruction, which occurs due to the formation of crystals within kidney tissue. The crystals form, grow, and then block the flow of urine in the kidney, which can lead to obstructive uropathy and renal failure. An observation of urinary crystals does not always correlate directly to renal toxicity, as has been shown previously for indinavir (Martinez et al. 1998), but more often than not, crystals observed in human urine are a cause for concern. Precipitation-based toxicity remains a relevant issue in pharmaceutical development due to the historical trend toward the selection of more receptor specific drug candidates having poor aqueous (i.e. urine) solubility (Lipinski 2000). Reduced hydrophilicity is often a by-product of increasing the receptor binding affinity of a compound; this is often accomplished at the molecular level by the addition of lipophilic groups at the sites of receptor interaction.

There has been significant past research addressing crystal and stone formation in the kidney and in urine, especially in relation to urine constituents which allow supersaturation of endogenous ions (Asplin et al. 1991, 1997; Coe et al. 1991; Hess et al. 1989). Other studies correlated crystal or stone formation with dietary parameters (Rotily et al. 2000; Borghi et al. 1999, 2002). These studies suggested that molecules may, depending on various factors, be present at supersaturated concentrations in urine. This past work, however, did not eliminate the concern that elevated urinary concentrations of M1 in study subjects could lead to crystalluria and/or toxicity. Therefore, a set of *in vitro* experiments to determine the solubility of M1 was initiated with the intention of directly relating these data to the solubility of this metabolite in the human urinary tract. A description of these experiments is contained herein.

2. Investigations, results and discussion

2.1. Room temperature solubility of M1 in drug-free urine

The solubility of M1 in control human and animal urine at room temperature was determined as shown in Table 1. Solid M1 was mixed with control urine for 48 h after which the samples were analyzed by HPLC. These results indicated that concentrations of M1 in urine, obtained following the administration of compound **1** to humans, shown in Figs. 1 and 2, had the potential for exceeding the room temperature urine solubility of the compound.

Our experience working with the urine collected during clinical testing has shown that storage at or below room temperature results in the precipitation of urine constituents. The consistency and degree of this precipitation varies greatly across urine samples from different subjects; variability is also often observed in samples from the same subject depending on the time of day that the urine was collected. These precipitants are known to be composed of a variety of components which include calcium,

Table 1: Urine solubility in various species of M1 after stirring for 48 hours at room temp

Species (n = 5)	Room temp solubility[a] (μg/mL) after 48 h
Male human	1.4 (31%)
Female human	1.3 (25%)
Male monkey	2.2 (23%)
Female monkey	1.2 (55%)
Male rat	6.2 (26%)
Female rat[b]	1.6 (43%)
Water[c]	0.6

[a] Numbers in parentheses are coefficients of variation
[b] There was no effect on solubility observed for urine adjusted to pH 2, 4, 6, 7, and 9
[c] n = 2

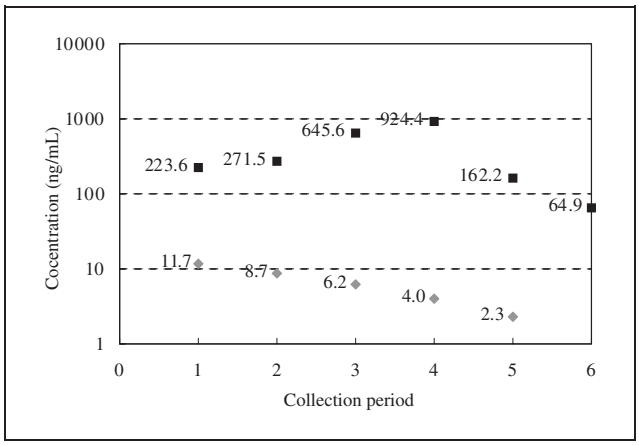


Fig. 1: Mean urine concentrations for compound **1** (◆) and its major hydroxylated metabolite, M1 (■) in elderly subjects after a single 6 mg oral dose. Collection intervals: (1) 0–2 h, (2) 2–4 h, (3) 4–8 h, (4) 8–12 h, (5) 12–24 h, (6) 24–48 h

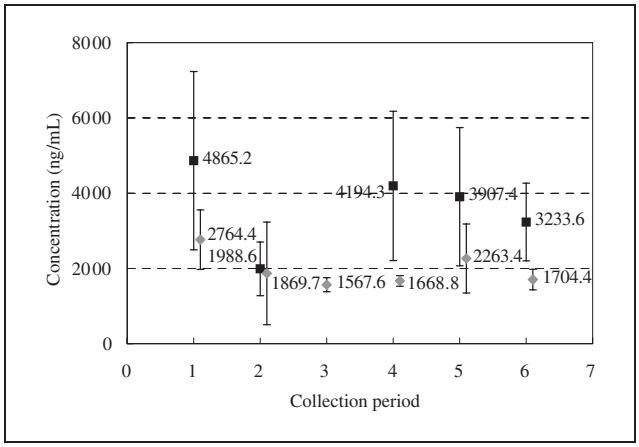


Fig. 2: Mean hydroxylated metabolite (M1) concentrations after the last dose of a multiple dosing regimen of compound **1** in the urine of elderly subjects (◆, 14 days, 6 mg, q8h) and young subjects (■, 10 days, 8 mg, q8h). Error bars represent ± one standard deviation of each mean. Collection intervals: (1) Predose, (2) 0–2 h, (3) elderly 2–4 h, (4) elderly 4–8 h, young 2–6 h (5) elderly 8–12 h, young 6–12 h (6) 12–24 h

uric acid, citrate and phosphate (Curhan et al. 2001), proteins including albumin (Cerini et al. 1999) and other miscellaneous compounds at smaller but significant concentrations. Many of these components could have some effect on either reducing or enhancing solubility of a compound in urine. It has been shown previously in our laboratories that urine from different human subjects can exhibit remarkably different solubilizing properties, and that the urine solubility of excreted drugs can be manipulated by the presence of albumin and other additives (Rose et al. 1999; Schwartz et al. 1997). Based on this, it was essential in these experiments to collect urine and maintain it without the precipitation of endogenous components, to provide a valid indication of the *in vivo* solubility of M1.

2.2. Solubility in human drug-free urine at body temperature

The next series of human urine experiments were a direct measurement of the solubility of M1 at body temperature. Precautions were taken to maintain the urine in its natural state; this included minimizing evaporation and storing the urine at 37 °C throughout the experiments. Urine was collected from human volunteers after 12 h of fasting. A second collection was made from the same subjects after drinking one liter of water. M1 solubility was then determined in each of the urine collections. As shown in Table 2, the mean concentrations of M1 were least variable at the 24 h time point (3556 to 3756 ng/mL). Mean concentrations and variability increased after 48 and 72 h of stirring. Concentrations of M1 in male versus female urine were not statistically different, except at the 72 h time point (t-test, $\alpha = 0.05$) where concentrations in male urine were higher. The solubility of M1 was not significantly (paired t-test, $\alpha = 0.05$) different in concentrated (osmolality 690 ± 198 mOsm/kg) vs hydrated urine (osmolality 182 ± 58 mOsm/kg).

These results gave considerable insight into the possibility of precipitation *in vivo* at higher doses of compound **1**. The primary goal of these experiments was to establish a margin of safety between the measured solubility of M1 and the levels of this metabolite that were observed in the urine of human clinical subjects. Establishing such a margin depended on finding that M1 concentrations in urine collected from subjects in clinical studies were consistently lower than the solubility of M1 determined in the *in vitro* experiments. No clear safety margin was identified between the solubility *in vitro* and the concentrations that were observed at the highest doses in the clinical study which ultimately limited the clinical dose range.

Some interesting subtleties did arise from the *in vitro* experiments. The first was that there was minimal effect on metabolite solubility due to the concentration of endogenous components in the urine. The average osmolality of the urine from fasted subjects was 690 ± 198 mOsm/kg compared to 182 ± 58 mOsm/kg for hydrated subjects. Despite these differences, neither was significantly better at solubilizing M1, although both were statistically better than water. Based on this, the conclusion can be made that the endogenous components potentially responsible for the increased solubility of M1 in urine relative to water are not measurably sensitive to moderate changes in urine hydration.

There remained some ambiguity in applying the solubility data obtained using drug-free human urine to the greater issue of the risk of dosing compound **1** to humans. From the outset, it was expected that the *in vitro* saturation solubilities would be considerably higher than the concentrations that were observed in clinical samples, given that no crystals were found in the urine of dosed subjects. The results of the *in vitro* experiments, however, indicated that there should be precipitation observed in at least a few of the subjects.

The results of a supersaturation experiment, shown in Fig. 3, offer a partial explanation for the lack of crystals in human urine, which at the highest clinical doses, should have been at or near supersaturation. In this experiment, distilled water was supersaturated with M1 at elevated temperature. The solubility of M1 increased to 20 μg/mL when the water was heated to 75 °C, showing the possibility for obtaining concentrations of M1 higher than those obtained in human urine during the *in vitro* experiments. It is conceivable that the kidney could also cause the supersaturation of M1 through the normal secretion process and that this supersaturation might be maintained during the residence time in the urinary tract. Evidence for such a phenomenon was shown by supersaturating water with M1 at 75 °C and then allowing this solution to sit at 25 °C for 15 h. In water, M1 was not observed to enter a phase of rapid precipitation after supersaturation, even in the presence of seed crystals of M1. The concentration of M1 at 25 °C, after supersaturation, remained almost five times greater than the concentration that could be obtained by stirring at 25 °C. This experiment presented strong evidence that in water, which was shown to be a poorer solvent for M1 than urine, the propensity for rapid precipitation from a supersaturated solution does not exist. Unfortunately, these experiments could not be duplicated effectively in urine, as heat denatures endogenous urine proteins and would have provided little urine-specific information.

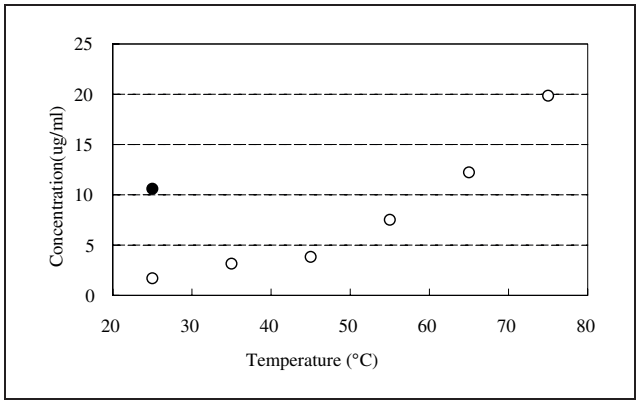


Fig. 3: Mean concentrations of hydroxylated metabolite, M1(○) at different temperatures in deionized water after mixing in sealed glass ampules. (●) indicates the concentration after shaking an ampule containing solid M1 at 75 °C for 24 h and then 25 °C for 15 h

Table 2: Solubility of M1 in drug-free human urine at 37 °C

Urine type	Concentration (ng/ml)		
	24 h[a]	48 h[a]	72 h[a]
All (n = 16)	3656 (17%)	4678 (25%)	5378 (46%)
Male (n = 8)	3680 (16%)	4985 (29%)	6980 (40%)
Female (n = 8)	3632 (18%)	4372 (17%)	3977 (22%)
Fasted (n = 8)	3756 (15%)	5245 (25%)	5179 (23%)
Hydrated (n = 8)	3556 (19%)	4112 (17%)	4906 (72%)
Water (n = 2)	1844	2240	2417

[a] Numbers in parentheses are coefficients of variation

2.3. Excretion of M1 in subjects administered compound 1

Following the administration of a single 6 mg oral dose of compound **1**, the maximum observed urine concentration was approximately 10 ng/mL of the parent drug and approximately 1000 ng/mL for the hydroxylated metabolite (M1). The concentration of M1 was consistently 10 to 100 times greater than that observed for parent over the 48 h that urine was collected (Fig. 1). Upon multiple administration of compound **1**, the highest mean concentrations of M1 were 4865 ± 2368 ng/mL and 2764 ± 791 ng/mL in healthy young subjects (8 mg, q8h, day 10) and healthy elderly subjects (6 mg, q8h, day 14), respectively (Fig. 2).

2.4. Solubility of M1 in urine from subjects dosed with compound 1

After determining a range for the solubility of M1 in drug-free control urine, further experiments were performed using urine collected from elderly subjects participating in a multiple dose clinical trial. The goals were to determine if the parent drug, additional metabolites, or other changes in the urine due to dosing had some effect on M1 solubility, and to establish some safety margin between the saturation concentration and the highest urine concentrations of M1 observed in clinical samples. Three possible outcomes were expected upon addition of solid to urine already containing M1 from metabolism. They included: 1) no change in M1 concentration, indicating that the urine was already or close to being saturated, 2) an increase in M1 concentration, indicating that the urine was not already saturated due to multiple dosing of the drug and 3) a decrease in M1 concentration providing evidence that the sample was already saturated due to multiple dosing and that addition of solid caused precipitation.

Table 3 shows the urine concentrations of M1 that were measured for each of the five subjects receiving the highest dose of active drug during the clinical study along with summary information for the three subjects that received placebo during the study. The mean concentration of M1 that was achieved by adding solid M1 to the urine of subjects receiving placebo was 2509 ng/mL with individual concentrations (not listed) ranging from 1288 to 3247 ng/mL. These subjects receiving compound **1** had significant concentrations of M1 in their urine (1830 to 6746 ng/mL). Upon addition of solid M1 to the urine of these subjects, the concentration of M1 was observed to rise in all cases, 3.2–83.0%, supporting the conclusion that a margin (albeit small for some subjects) existed between the clinical concentrations and concentrations at which saturation and precipitation would occur.

The margin to saturation was shown to be highly dependent on the initial concentration of M1; urine starting out at higher concentrations increased by a much lower percentage than urine containing lower initial concentrations.

Ultimately, the impact of the data was tempered by the overlap between experimental and clinical concentrations and would have been much more definitive had some consistent safety margin been identified. In the end, this data limited the dose of compound **1** that could be administered during clinical studies.

Table 3: Solubility of M1 at 37 °C in human urine collected from subjects receiving compound 1 orally (14 days, q8h)

Subject	Day	Control (ng/mL)	Solid added (ng/mL)	% Increase
1	4	5158	5466	6.0
	7	6746	7279	7.9
	10	3831	4212	10.0
2	4	2162	3957	83.0
	7	2895	3929	35.7
	10	1830	3087	68.7
3	4	4758	4910	3.2
	7	4858	5191	6.9
	10	5294	5987	13.1
4	4	3300	4679	41.8
	7	2307	3394	47.1
	10	2559	3447	34.7
5	4	2346	3206	36.6
	7	3486	4385	25.8
	10	2721	3859	41.8
Mean concentration (ng/mL)[a] and % increase				
Day 4		3545 (39%)	4443 (20%)	25.3
Day 7		4058 (44%)	4835 (31%)	19.2
Day 10		3247 (42%)	4118 (27%)	26.8
Day 4–10		3617 (40%)	4466 (26%)	23.5
Placebo (n = 3)		NA	2509 (22%)	NA

[a] Numbers in parentheses are coefficients of variation

3. Experimental

3.1. Materials

Compound **1**, M1, and internal standard were obtained from Merck Neuroscience Research Center (Terlings Park, UK). Solvents were obtained from EM Science (Gibbstown, NJ, USA). Ammonium acetate was obtained from Aldrich (Milwaukee, WI, USA). All other chemicals were obtained from Fisher Scientific (Springfield, NJ, USA).

3.2. HPLC-MS/MS assay instrumentation and parameters

The bioanalytical method used to determine compound **1** and M1 was validated in accordance with generally accepted criteria (Shah et al. 2000) and was applied to the determination of compound **1** and M1 as follows: The HPLC system consisted of a Perkin Elmer LC200 pump (Norwalk, CT, USA) and a Varian Prostar 430 autosampler (Walnut Creek, CA, USA). The mass spectrometer was a Sciex API 3000 triple quadropole HPLC-MS/MS (Foster City, CA, USA) with a heated nebulizer interface (500 °C). PE Sciex MacQuan software was used to collect and process the data. The mobile phase consisted of 35/65 (v/v) acetonitrile/10 mM ammonium acetate, adjusted to pH 4 with acetic acid at a flow rate of 0.7 mL/min. Chromatography was performed on a 4.6×100 mm Restek (Bellefonte, PA, USA) Ultra IBD column with a 10×4.0 mm Ultra IBD guard. A representative chromatogram is shown in Fig. 4 for the analysis of compound **1**, M1, and the internal standard used in the assay. Multiple reaction monitoring of the precursor to production pairs m/z $363 \rightarrow 268$ for compound **1**, m/z $379 \rightarrow 284$ for M1, and m/z $377 \rightarrow 268$ for the internal standard, was used for quantitation.

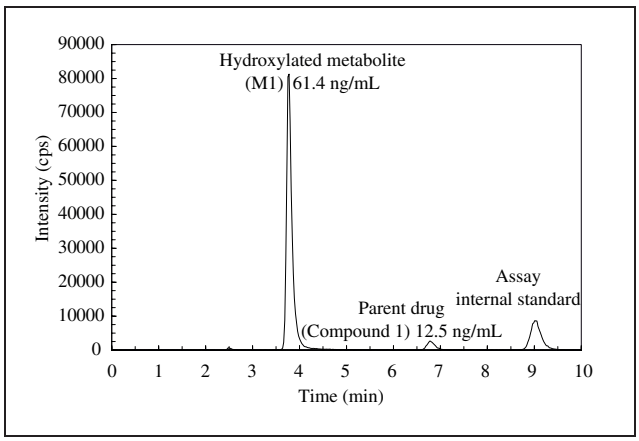


Fig. 4: Total ion HPLC-MS/MS chromatogram showing parent drug (compound **1**), its major hydroxylated metabolite (M1), and the internal standard used in the bioanalytical assay

Analytes were extracted from urine samples (0.5 mL) using a 3M Empore C_8 extraction plate, eluted with 325 μL of mobile phase and injected (30 μL) onto the HPLC-MS/MS. Concentrations of compound **1** and M1 in clinical samples were determined from a standard curve prepared daily by adding stock working standards into 0.5 mL of drug-free human urine. The range of the standard curve was 1 to 1000 ng/mL for each analyte. Accuracy for the determination of compound **1** and M1 (1 to 1000 ng/mL) in five different lots of human urine was within 98.0 to 102.3% and 96.2 to 104.5% of expected, respectively, while precision was 1.9 to 4.9% and 2.3 to 8.2%, respectively. Quality control (QC) samples in human urine, containing compound **1** and M1, were prepared at concentrations of 3, 250, and 900 ng/mL and stored under the same conditions as the samples for a period longer than the duration of the study. These QCs were quantitated along with the samples to verify analyte stability and assay accuracy. Compound **1** and M1 were stable in human urine at these concentrations throughout the analytical testing procedure, including up to three freeze-thaw cycles (freezing at −20 °C and thawing at room temperature). The ability to accurately quantitate M1 in urine at concentrations greater than the assay upper limit of quantitation (ULOQ) after a single freeze thaw cycle was demonstrated. Urine samples were thawed, diluted 1 : 10 with drug free control urine, and assayed for M1 as described above. The average accuracy obtained for these samples was within 1% of nominal.

3.3. Solubility experiments in water and urine

3.3.1. Room temperature solubility studies

Unfiltered urine (3 mL) freshly obtained from healthy human subjects was added to a 4-mL glass vial containing solid M1 (3 ± 0.2 mg). The vial was capped, and mixed using a rotary mixer for 48 h at room temperature. After 48 h, the concentration of M1 was measured by HPLC.

3.3.2. Supersaturation studies in water

Water (2 mL) was added to glass ampules (5 mL) containing 2.1 ± 0.2 mg of M1 solid. The ampules were sealed using a flame and were placed on a Vobromixer (Tehtnica, Zelezniki, Slovenia) for 2 h at temperatures of 25, 35, 45, 55, 65, and 75 °C (3 vials at each temperature). After mixing, the ampules were centrifuged (2 min, 2500 × g), opened and analyzed. To characterize the return to equilibrium concentration, an ampule at 75 °C was kept sealed and allowed to remain at 25 °C for 15 h after which it was centrifuged, opened, and analyzed.

3.3.3. Solubility in drug-free human urine at 37 °C

Four male and four female subjects provided urine using a clean-catch procedure after fasting for 12 h. An additional specimen was obtained from these subjects one hour after drinking 1 L of water. These urine samples were designated as "concentrated" and "hydrated" urine, respectively. A 2-mL aliquot was removed from each urine sample and added to a 4.5 mL Nalge Nunc cryogenic tube (Naperville, IL, USA) containing 1 ± 0.1 mg of solid M1. The remainder of the urine sample, for use in diluting samples during the experiment, was capped and maintained at 37 °C in an environmental chamber from Thermo Forma (Marietta, Ohio, USA). The tubes containing urine and M1 solid were mixed horizontally (150 rpm, 37 °C) in an Innova 4300 incubated shaker (New Brunswick Scientific, Edison, NJ, USA) for 3 days. At 24, 48 and 72 h, the Nunc tubes were removed from the shaker, placed in a temperature controlled (37 °C) aluminum block and a 200 μL aliquot was removed from each tube and placed into 1.4 mL eppendorf centrifuge tube also under controlled temperature. These tubes were centrifuged in an Eppendorf Mini Spin Plus (Eppendorf Brinkman, Westbury, NY, USA) contained within the 37 °C environmental chamber (13,000 × g), to remove undissolved drug from solution, prior to sampling. An aliquot (50 μL) was pipetted from the upper supernatant and added to 4.95 mL of the corresponding control urine. A similar experiment was performed using water in place of urine in order to obtain solubility data in this matrix. The diluted sample was mixed well and then frozen at −20 °C prior to analysis using HPLC-MS/MS. Urinalysis of concentrated and hydrated control urine was performed on day 1 to obtain values for osmolality and pH. The pH values for all urine were within the normal range of 5 to 7.

3.3.4. Solubility in the urine of subjects receiving study medication

To characterize the saturation solubility of the M1 in fresh human urine from subjects dosed with compound **1**, eight elderly subjects were administered 6 mg of compound **1**, orally, every 8 h (q8h) for 14 days as part of a clinical study to evaluate the tolerability and pharmacokinetics of compound **1** in healthy elderly subjects. On Day 1, control, drug-free urine was collected from each subject and frozen at −20 °C. Urine samples were then collected at 2 to 4 h after a dose of compound **1** administered on days 4, 7, and 10. Physiological temperature (37 °C) was maintained throughout the experiment, including the use of pre-warmed pipet tips. Aliquots (2 mL) of the urine collections were transferred into each of two 4.5 mL Nalge Nunc cryogenic tubes; one of the tubes was empty while the other contained 1 ± 0.1 mg of solid M1. These tubes were tightly capped, transferred to a rotary mixer and mixed at 1400 rpm, 37 °C, for 1 h. The tubes were removed after 1 h of mixing and were centrifuged at 13,000 × g for 10 min. A 50-μL aliquot was removed from the top of the supernatant and transferred to a 15 mL centrifuge tube already containing 4.95 mL of the corresponding control urine that had been collected at the start of the study. The samples were well mixed, frozen at −20 °C and shipped on dry ice to the laboratory for analysis by HPLC-MS/MS.

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