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Original research paper

Miniaturized shake-flask HPLC method for determination of distribution coefficient of drugs used in inflammatory bowel diseases

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A new method for determination of distribution coefficient of drugs azathioprine, 6-mercaptopurine and 6-thioguanine and nutrient folic acid used in the treatment of inflammatory bowel disease based on a miniaturized shake-flask and HPLC/DAD was developed. Special attention was made to the most commonly reported problems in the measurement of distribution coefficients using a shake-flask method such as mixing technique, speed and time, the temperature of experiment, type of buffer and its pH as well as *n*-octanol/buffer phase ratio. The concentration of compounds in the buffer is determined by HPLC directly from shake flasks or conventional 2-mL vials. The developed method was fully validated according to ICH guidelines. Furthermore, experimental data were successfully compared with lipophilicity and human intestinal absorption calculated by the use of four different theoretical approaches. The method shows potential for high-throughput measurements of a large number of compounds.

Keywords: distribution coefficient logarithm, shake-flask, HPLC, *in silico*, inflammatory bowel disease

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Lipophilicity represents one of the most studied and most frequently used fundamental physicochemical property that affects compound solubility, determines its passive transport through biological membranes, influences biodistribution, metabolism, elimination and toxicity.

A large variety of procedures for lipophilicity determination were developed. These methods can be divided into two major groups, direct and indirect ones. In the direct methods, the shake-flask and stir-flask procedures are included, whereas various separation methods represent the indirect ones. Because of its simplicity and clear relationship to the partitioning phenomenon, the traditional shake-flask technique is regarded as a benchmark method against which other methods are validated. It consists of partitioning of the analyte in *n*-octanol and water (buffer) mixture, and afterward measuring the concentration of the

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analyte in each phase. The lipophilicity of the analyte is expressed throughout logarithm of partition coefficient, log *P*, or for ionizable analytes throughout logarithm of distribution coefficient at certain pH, log D_{pH}. Shake-flask procedure is applicable for hydrophilic and lipophilic compounds, as well as neutral and charged analytes. On the other side, it is time and labor consuming, requires relatively large amounts of pure compounds, complete solubility of analyte must be attained and *n*-octanol-water emulsions can cause severe problems. Furthermore, the accurate measurements of the partition and distribution coefficients require the use of highly sensitive and precise analytical methods (1).

In order to avoid these inconveniences, modifications of shake-flask methods have been published. These investigations were focused on the improvement of shake-flask performance in terms of reproducibility and lowering manual work during sample preparation procedures and/or finding more sensitive and selective analytical methods than the most commonly used UV-Vis spectrophotometric technique.

One of the major disadvantages of the shake-flask method is a long sample preparation procedure. Therefore, Wenlock and co-workers (2) have proposed a modified shake-flask procedure for simultaneous measurement of the distribution coefficients of a mixture of up to 10 compounds using high-performance liquid chromatography and tandem mass spectrometry (LC/MS/MS). During the determination of partition and distribution coefficients of a highly hydrophobic compound, the concentration of the compound in *n*-octanol phase is much higher than that in the water phase, making it difficult to separate water and *n*-octanol phases without the contamination of water phase. To resolve this problem, a new water plug aspiration/injection method was successfully applied for the determination of distribution coefficient of highly hydrophobic compounds (3). Afterwards, the same group of authors also introduced an automated liquid-handling system to increase the throughput of measurements (4). Finally, several reports have shown remarkable improvements in the automatization and increase of throughput by using 96-well plates (3–6). In drug development, low amounts of test compounds are available for preclinical studies, thus Andrés and co-workers (7) have designed a shake-flask procedure for the determination of distribution coefficient from low drug amounts.

Traditionally, lipophilicity measurements were performed using a UV-Vis spectrophotometer (8–10). This technique suffers from low sensitivity and is not easily converted to a high-throughput format. Over the past decade, a number of studies have been taken to improve this assay. Numerous literature reports have shown a prominent place of the HPLC technique (7, 11–13). In recent years, hyphenated techniques (LC/MS and LC/MS/MS) have received ever-increasing attention due to superior selectivity and sensitivity of MS detector (2–5, 14). To improve the accuracy of the method, Nishimura and co-workers (6) have introduced the injection marker into a shake-flask procedure followed by LC/MS analysis. Other separation techniques, such as thin-layer chromatography (15), ultra-performance liquid chromatography (16) and capillary zone electrophoresis (17), have also been employed to achieve increased throughput, sensitivity, precision and accuracy of the determination of partition and distribution coefficients.

The term inflammatory bowel disease (IBD) describes two well-established, but not completely distinguished disease entities: ulcerative colitis and Crohn's disease. These conditions comprise of immune-mediated inflammatory processes occurring in genetically predisposed individuals. It is presumed that the environmental triggers react with the intestinal flora, thus causing inflammation in the gastrointestinal tract (18), leading to diarrhea, abdominal pain and nutrient malabsorption among others (19). These conditions

are treated with a broad spectrum of drugs: corticosteroids and monoclonal antibodies, such as infliximab, are used for more severe cases, whereas aminosalicylate mesalazine with its prodrugs and purine analogues azathioprine (AZA), 6-mercaptopurine (6-MP) and 6-thioguanine (6-TG) are helpful in the maintenance of the remission. Patients are also often prescribed folic acid (FA) for the treatment of megaloblastic anemia caused by the aforementioned malabsorption.

Fixed-dose combinations (FDC) improve patient adherence and should be considered in patients with chronic diseases (20), therefore a FDC of FA and one of the purine analogues could be useful in the treatment of IBD. Prior to the development of novel FDC, a complete insight into physicochemical properties of active pharmaceutical ingredients (API), one of which is lipophilicity, is needed in order to evaluate the interactions of drugs with each other, as well as with the human organism (21). To best of our knowledge, the lipophilicity of these compounds was investigated using indirect chromatographic techniques such as thin-layer chromatography (22, 23), as well as using the traditional shake-flask procedure with UV-Vis spectroscopy (24, 25).

In the spirit of above considerations, the purpose of this work was to optimize a miniaturized shake-flask high-performance liquid chromatography with diode-array detector (HPLC/DAD) method for the determination of distribution coefficients of AZA, 6-MP, 6-TG and FA (Fig. 1).

An attempt has been made to develop an automatic sampling method instead of a manual separation of two phases to increase the throughput of measurements and to minimize the manual interventions. The aim of our work was to present and discuss each method optimization step and to validate the proposed method in order to improve the reproducibility and accuracy of the measurements. A further purpose of this study was to assess the comparability of the experimental and theoretical results as well as to relate the experimental log $D_{7.4}$ values with pharmacokinetic parameters, specifically human intestinal absorption (HIA) of investigated compounds.

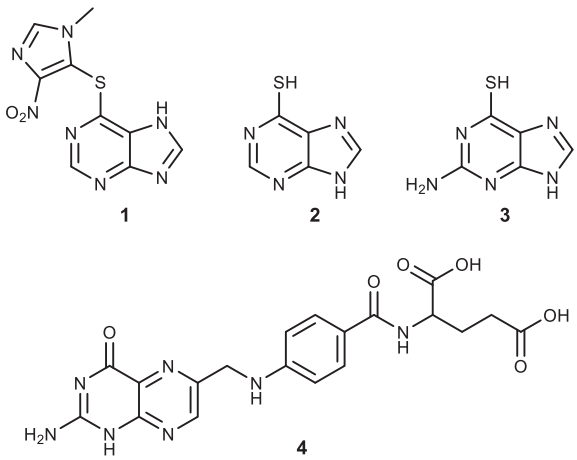

Fig. 1. Structures of investigated compounds: AZA (1), 6-MP (2), 6-TG (3) and FA (4).

EXPERIMENTAL

Materials

AZA, 6-MP, 6-TG, FA, dimethylsulfoxide and phosphate buffered saline (PBS) tablets, all HPLC grade, were obtained from Sigma Aldrich (Germany). Acetonitrile, HPLC grade, was obtained from J. T. Baker (Deventer, The Netherlands). Formic acid, HPLC grade, was purchased from Merck (Germany). *n*-octanol, purified, was obtained from VWR (France). Ultrapure water was prepared with a Mili-Q water purification system (Millipore, USA).

Methods

Shake-flask determination

Distribution coefficients were determined using pre-saturated solutions of *n*-octanol and 10 mmol L^{-1} PBS, pH 7.4. To achieve the saturation of PBS with *n*-octanol and *vice versa*, solvents were mixed as 1:1, stored for 24 h in an orbital shaker-incubator ES-20/60 (Biosan, Latvia) at 100 oscillations per min at 25.0 ± 0.1 °C. The prepared mixture was left overnight for phase segregation in the thermostat at 25.0 ± 0.1 °C.

Stock solutions of AZA, 6-MP and 6-TG were prepared using PBS pre-saturated with *n*-octanol in the concentration of 20 μg mL^{-1}. A stock solution of FA was prepared using dimethylsulfoxide in the concentration of 2000 μg mL^{-1}. Working solutions were prepared daily by dilution of the stock solutions with PBS pre-saturated with *n*-octanol.

0.15 mL of working solution was added to 1.5 mL of *n*-octanol pre-saturated with PBS in amber 2-mL HPLC vials. The samples were then mixed in the mechanical shaker at 200 oscillations per min for 1 h at 25.0 ± 0.1 °C. Afterward, the samples were centrifuged using a Z 326 K table top centrifuge (HERMLE Labortechnik, Germany) for 30 min at 4000 *g* and 25 °C to remove possible emulsions. The sample analyses were performed using the liquid chromatographic system Agilent 1100 (Agilent Technologies, Germany) and the chromatographic column ZORBAX SB-C8, dimensions 150 × 4.6 mm, particle size 5 μm with a suitable guard column ZORBAX SB-C8, dimensions 12.5 × 4.6 mm, particle size 5 μm both purchased from Agilent Technologies. The elution was carried out using ultrapure water with 0.1 % (*V*/*V*) formic acid as a mobile phase A and acetonitrile with 0.1 % (*V*/*V*) formic acid as a mobile phase B with a gradient program, starting with 5 % of the mobile phase B, ramping up linearly to 20 % until 3 min, and once again ramping up linearly to 55 % until 7 min. Additional 3 minutes were needed to re-equilibrate the column. Both mobile phases were filtered through a membrane filter, no. 66, diameter 47 mm, pore size 0.45 μm (Supelco, USA). The column temperature was set to 25.0 ± 0.1 °C and the flow rate of 1.0 mL min^{-1}. The vials were placed in a rack on the autosampler at 25 °C. The injection volume was 10 μL, with a needle offset of 0.5 mm. The absorbance of the analytes during a chromatographic run was collected in the spectral range of 200–400 nm. The detection wavelength was the one providing the maximum peak height, that is, 280 nm for FA and AZA, 320 nm for 6-MP and 343 nm for 6-TG. The sample from the lower buffer phase was injected for HPLC analysis before and after equilibration with the *n*-octanol phase. All measurements were done in duplicate.

The log $D_{7.4}$ values were calculated *via* equation *1*:

$$\log D_{7.4} = \log \left[\left(\frac{c_{0\mathrm{PBS}}}{c_{\mathrm{PBS}}} - 1 \right) \times \frac{V_{\mathrm{PBS}}}{V_{n-\mathrm{octanol}}} \right] \qquad (1)$$

where c_{0PBS} and c_{PBS} were concentrations of the analyte in the buffer phase prior to and post equilibration and V_{PBS} and $V_{n\text{-octanol}}$ were the volumes of buffer and *n*-octanol phase, respectively.

In silico *prediction*

Four online-available computer platforms for the evaluation of physicochemical and ADME parameters were used. List of predictors, lipophilicity indices and pharmacokinetic properties is given in Table I.

Table I. List of lipophilicity and pharmacokinetic property predictors

Program	Parameter	Abbreviation	Website
ADMETLab	distribution coefficient human intestinal absorption	$\log D_{Lab}$ HIA_{Lab}	www.admet.scbdd.com
Chemicalize	distribution coefficient	$\log D_{Chemicalize}$	www.chemicalize.com
pkCSM	human intestinal absorption	HIA_{pkCSM}	www.biosig.unimelb.edu.au/pkcsm/
PreADMET	distribution coefficient human intestinal absorption	$\log D_{PreADMET}$ $HIA_{PreADMET}$	www.preadmet.bmdrc.kr

Statistical analysis

Statistical analysis was carried out using the statistical package Microsoft Office Excel 2007 (Microsoft, Redmond, USA).

RESULTS AND DISCUSSION

Method development

Distribution coefficients obtained by the shake-flask method are strongly influenced by experimental conditions, thus optimization of experimental conditions was carefully performed. The optimization of the method was carried out using a univariate optimization approach. Different parameters influencing the distribution of analytes in *n*-octanol and buffer phases, including mixing technique, speed and time, the temperature of the experiment as well as phase ratio, were optimized with the one-variable-at-a-time method.

Several mixing techniques were investigated: vigorous shaking using a vortex and gentle mixing using a shaker and a stirrer. Vortex mixing was found to be the least time-consuming. Still, both layers appeared cloudy even after leaving overnight. Therefore, gentle mixing was preferred to avoid emulsion formation. Visual inspection of post-mixing layers obtained by the shaker and the stirrer indicated clear layers of *n*-octanol and buffer. Another advantage of these sample preparation techniques is that the temperature of samples can be easily controlled. Both techniques gave reproducible data, still due to practical reasons for our laboratory, as the less manual intervention was needed, the mechanical shaker was chosen for further study.

An important experimental parameter is shaking speed. Therefore, the partition of 6-MP as a representative of purine analogues and FA was examined by varying shaking speed from 50 to 200 oscillations per minute. The obtained results have shown that the equilibration was not achieved at low shaking speeds, thus, 200 oscillations per minute were chosen for further experiments. To determine the optimum time required to shake the samples in order to reach the equilibrium, an experiment was conducted in which the sampling of the buffer phase for partition of 6-MP and FA was performed as a time-course. Therefore, the concentration of analytes in both phases was determined after 0.5, 1, 6 and 12 h. Shaking time sufficient for the equilibration to be reached in HPLC vial was 1 h. Furthermore, it was important to evaluate the stability of analytes in the *n*-octanol/buffer mixtures. According to our findings, the compounds of interest were stable in the buffer phase for at least 1 h, as no peaks other than that of the main compound were observed.

It is well known that the distribution coefficients of compounds strongly depend on the temperature of the system (26). However, during the testing of the influence of the temperature on the distribution coefficients, it has been observed that FA was susceptible to thermal degradation, which is in agreement with the results of Delchier *et al.* (27). According to these findings, a temperature of 25 °C was chosen for further experiments. To minimize the influence of temperature all experiments were performed in well-controlled temperature conditions; samples were shaken in a thermostat at 25.0 ± 0.1 °C and were kept in HPLC autosampler at 25 °C until the analysis.

Regarding the buffer used for the procedure, PBS was chosen to mimic physiological conditions. Furthermore, UV transparency makes it ideal for HPLC analysis.

To obtain the satisfying reproducibility of the method, various phase ratios were tested. Distribution coefficients were determined under different test conditions, whereby the ratios of buffer and *n*-octanol phase were varied: 10:1, 1:1 and 1:10. Best reproducibility of the results was obtained at 1:10 ratio, which was chosen for further experiments.

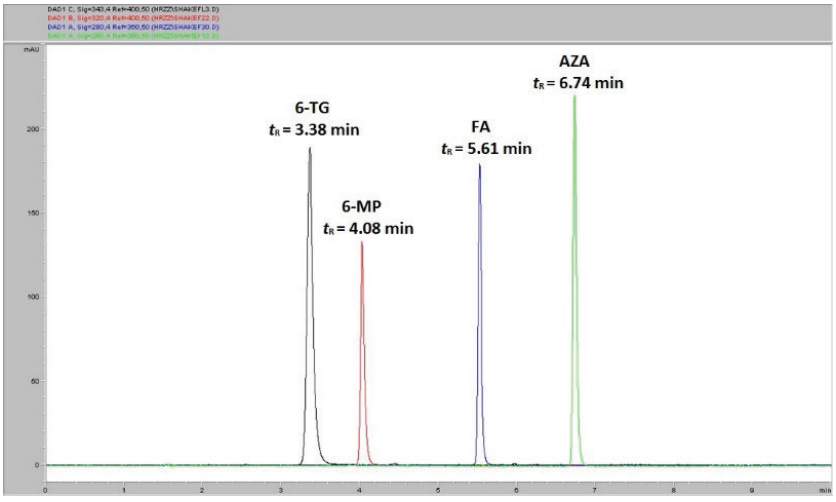

Fig. 2. Overlaid chromatograms of 6-TG (black), 6-MP (red), FA (blue) and AZA (green) with corresponding retention times (t_R).

Among different techniques available, HPLC has dominated the separation, identification and quantitation of a broad spectrum of compounds in the last thirty years. Several stationary and mobile phases were used for the analysis of FA and purine analogues. Most of the chromatographic methods include the use of, almost exclusively, reversed-phase chromatographic columns C18 and C8, detectors such as UV-Vis or, if necessary, MS and mobile phases containing mixtures of polar organic solvent and acidified water (28–30). Therefore, the analysis of our samples was performed using a Zorbax SB-C8 column that can be used at low pH without degradation. To achieve a high-throughput analysis time of less than 7 min, gradient elution was used. The optimized conditions yielded symmetrical and sharp peaks of all the analytes without peak tailing (symmetry factors were above 0.8, theoretical plate count was more than 8477) (Fig. 2).

Validation of the method

As reported before (8, 9), the major disadvantage of the shake-flask method is unsatisfactory reproducibility and reliability of obtained partition and distribution coefficients. Thus, careful validation of the proposed method was performed to improve the quality and reproducibility of the obtained data. The method validation was performed following the recommendation of International Conference on Harmonization of Technical Requirements for Registration of Pharmaceuticals for Human Use (31). The method was validated for selectivity, linearity, accuracy, sensitivity, precision, stability and robustness.

To evaluate the presence of potential interferences from the buffer phase, the sample to which no analyte had been added was evaluated. The obtained chromatogram reveals that no other components were co-eluted with compounds of interest (peak purity factors were higher than 999.7).

Linearity of the shake-flask method was estimated using five concentration levels for each analyte (0.5, 2.5, 10, 15 and 20 μg mL^{-1}). The obtained correlation coefficients (r) were higher than 0.999 indicating excellent linearity of the method (Table II). Since small amounts of the lipophilic compounds were expected to be found in the buffer phase, sensitivity of the optimized method was one of the most important validation parameters. Limits of detection (*LOD*) and quantitation (*LOQ*) were determined on the basis of signal-to-noise ratios (S/N) of 3:1 and 10:1, respectively. As it can be seen from Table II, the method was proven to be quite sensitive for the determination of small concentrations of all analytes ($LOQ \leq 0.50$ μg mL^{-1}).

Precision and accuracy of the method were also evaluated. The analysis of the samples (10 μg mL^{-1}) was repeated six times within the same day to determine repeatability

Table II. Method calibration data

Analyte	Linearity correlation range (μg mL^{-1})	Equation	Correlation coefficient (r)	*LOD* (μg mL^{-1})	*LOQ* (μg mL^{-1})
AZA	0.50–20	$y = 31.60\ x - 9.75$	0.9995	0.14	0.46
6-MP	0.50–20	$y = 68.20\ x - 17.61$	0.9997	0.07	0.23
6-TG	0.50–20	$y = 72.21\ x - 16.61$	0.9998	0.13	0.42
FA	0.50–20	$y = 25.47\ x - 4.44$	0.9999	0.15	0.50

Table III. Precision and accuracy of the method

Analyte	Intra-assay precision RSD (%)	Inter-assay precision RSD (%)	Accuracy recovery (%) ± RSD (%)		
			low	medium	high
AZA	0.71	0.82	100.22 ± 0.19	100.21 ± 0.71	99.84 ± 0.16
6-MP	0.58	0.91	99.91 ± 1.10	100.76 ± 0.75	99.32 ± 1.11
6-TG	0.41	0.38	99.34 ± 1.21	100.46 ± 0.66	99.15 ± 0.14
FA	0.57	1.23	97.45 ± 0.36	100.71 ± 0.54	99.84 ± 0.16

(intra-assay precision) and three times on two different days to determine intermediate precision (inter-assay precision) of the method. The obtained Relative Standard Deviation (RSD) values were lower than 0.71 % and 1.23 %, confirming high repeatability and intermediate precision of the method (Table III). To determine the accuracy of the chromatographic method, samples were analysed in triplicate on low (2.5 μg mL^{-1}), medium (10 μg mL^{-1}) and high (20 μg mL^{-1}) concentration levels. All recoveries were in the range of 97.45 to 100.76 %, confirming good accuracy of the method (Table III).

Robustness of the chromatographic method was also evaluated. Deliberate minor changes to the column temperature, gradient program and he mobile phase composition were made. RSD values of peak areas and retention times (t_R) prior to and post changes were lower than 4.34 and 7.15 %, respectively.

As a part of the method validation, data were also generated to ensure that all analytes were stable at distinct timing and temperature conditions. Stability tests were performed in terms of short-term (at room temperature for 8 hours) and long-term storage (in the refrigerator at –20 °C for 7 days) in standard solutions (10 μg mL^{-1}). The percentage of analytes recovered from the samples ranged from 97.5 to 99.5 %. These results indicate that the degradation of all analytes was not significant during chosen conditions.

Determination of distribution coefficients

The optimized miniaturized shake-flask method was successfully applied for the determination of distribution coefficients of AZA, 6-MP, 6-TG and FA (Fig. 1). AZA, 6-MP and 6-TG belong to the group of thiopurines. They all share a purine skeleton, which is comprised of a pyrimidine ring fused to an imidazole ring, with a sulfur atom bonded at position C6, this also being the structure of 6-MP. 6-TG additionally has an amino group at position C2, whereas AZA has 1-methyl-4-nitroimidazole ring bound to sulfur. FA, on the other hand, consists of three large sub-components: 2-amino-4-oxopteridine joined by a methylene bridge to *p*-aminobenzoic acid, which is connected by an amide bond to L-glutamic acid.

The method was capable of measuring log $D_{7.4}$ values from –3.65 to 0.08 (Table IV). Furthermore, RSD values lower than 0.89 % indicated high reproducibility of the method. As was expected, distribution coefficients were related to the chemical structure of the compound. AZA and 6-TG had very similar log $D_{7.4}$ values; however, AZA appeared to be slightly more lipophilic, possibly due to the presence of the large imidazole derivative ring.

Although 6-MP and 6-TG are very structurally similar, 6-MP appears to be more hydrophilic, presumably due to a very small percentage of amino groups at position C2 of 6-TG being ionized at pH 7.4, thus increasing overall lipophilicity. The trend of increasing lipophilicity in the order of 6-MP < 6-TG < AZA is in accordance with results of Chrzanowska *et al*. (24, 32), whose experimentally measured partition coefficients at pH 7.4 followed the same order. Log $D_{7.4}$ of FA is, however, significantly more negative than that of thiopurines, implying higher hydrophilicity. This could be due to the fact that FA contains a larger number of ionizable groups than the thiopurines, few of which are ionized at set pH, contributing to the overall negative charge of the molecule. Enyedy *et al.* (25) also came to similar conclusions regarding lipophilicity of FA while researching *n*-octanol-water distribution coefficient of FA and its derivatives by UV-Vis spectroscopy. The insignificant difference of absorbance of aqueous solution of FA before and after equilibration was found ($\Delta Abs < 0.005$ at λ_{max}), implying very high hydrophilicity.

Comparison of experimental and predicted data

During the last several years, a number of calculation procedures were developed for rapid estimation of molecular lipophilicity (8, 9, 25). While many calculation procedures have been developed for rapid estimation of log *P* values, only several are available for prediction of distribution coefficient at various pH values. Experimentally obtained log $D_{7.4}$ values were compared with the ones predicted by three calculation platforms: Chemicalize, PreADMET and ADMETlab (Table IV). Predicted log $D_{7.4}$ values for

Table IV. Experimental and predicted log $D_{7.4}$ values for the investigated compounds and differences between experimental and predicted values for Chemicalize, PreADMET and ADMETLab platforms

Analyte	Experimental log $D_{7.4}$ ± RSD (%)	Chemicalize		PreADMET		ADMETLab	
		log $D_{Chemicalize}$	Δlog $D_{7.4}$	log $D_{PreADMET}$	Δlog $D_{7.4}$	log D_{Lab}	Δlog $D_{7.4}$
AZA	0.08 ± 0.55	1.15	1.07	0.67	0.59	0.11	0.03
6-MP	–0.26 ± 0.09	–0.12	0.13	–0.47	0.22	–0.04	0.21
6-TG	0.06 ± 0.89	–0.35	0.41	–0.35	0.41	–0.09	0.15
FA	–3.65 ± 0.30	–6.93	3.29	–0.59	3.05	–0.59	3.05

Table V. Human intestinal absorption data for all analytes predicted by pkCSM, ADMETLab and PreADMET platforms

Analyte	HIA_{pkCSM} (%)	HIA_{Lab} (%)	$HIA_{PreADMET}$ (%)
AZA	78.6	78.2	75.5
6-MP	91.0	81.3	87.8
6-TG	91.0	76.7	83.1
FA	2.3	39.8	23.2

thiopurines for all of the platforms are somewhat in agreement with the experimental values, with ADMETLab platform showing the lowest difference between experimental and predicted values (Δlog $D_{7.4}$ values were lower than 0.21). Predicted log $D_{7.4}$ values for FA, on the other hand, ranged from –6.93 to –0.59, as compared to the experimental value of –3.65. As can be seen from the results, some platforms underestimate the lipophilicity of FA, while others overestimate it. This could be due to the fact that the platforms do not take the number of different ionizable groups, as well as the overall charge of such molecule, into account. These results may imply that more insight is needed in predicting lipophilicity parameters for more complex molecules.

HIA has often been connected to the lipophilicity of xenobiotics (33), therefore the selected parameter was predicted by pkCSM, PreADMET and ADMETLab platforms (Table V). According to obtained data, thiopurines are likely to be easily absorbed through the gastrointestinal tract, with predicted HIA percentages ranging from 75.5 to 91.0 %, while all of the platforms predicted poorer absorptions for FA (2.3 to 39.8 %). Seeing that pH in the small intestine is relatively neutral and thus close to 7.4 (34), these results can be correlated with the experimentally obtained log $D_{7.4}$ values. The highly hydrophilic FA is less likely to pass the intestinal membrane by passive transport, thus having lower HIA percentages, unlike the more lipophilic thiopurines. A number of studies, however, determined the absorption of FA to be relatively high, ranging from 75 to 90 % (35). It can be concluded that the platforms predict HIA according to lipophilicity, but do not take the vital role of various transporters present in the human intestines into account. This approach consequently lowers the prediction accuracy. HIA is a very complex process and it seems that *in silico* predictions still have a long way to go in establishing such algorithms.

CONCLUSIONS

The miniaturized shake-flask followed by HPLC/DAD method for the determination of distribution coefficients of drugs and nutrient used in therapy of inflammatory bowel disease has been developed. Sample preparation has been simplified and accelerated through the use of conventional HPLC 2-mL vials as a flask. The obtained experimental log $D_{7.4}$ values were compared with theoretical data obtained by 4 commercially available or free on-line physicochemical and pharmacokinetic property predictors. The miniaturized shake-flask procedure followed by HPLC/DAD analysis has proven to be fast, effective and, once optimized and validated, shows potential for high-throughput measurements of a large number of compounds. The obtained results may be useful for all researchers involved in the field of the determination of molecular lipophilicity.

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