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Experimental determination of drug diffusion coefficients in unstirred aqueous environments by temporally resolved concentration measurements

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\[ t_0 \]

\[ t_1 \]

\[ t_2 \]

\[ \text{Compound molecules} \]

\[ \text{Diffusion media} \]

\[ \text{Abs} \]

\[ t_0 \rightarrow t_1 \rightarrow t_2 \rightarrow t_\text{f} \]

\[ \text{time} \]

\[ \downarrow \]

\[ \text{Diffusion coefficient} \]
Abstract

The diffusion coefficient (also known as diffusivity) of an active pharmaceutical ingredient (API) is a fundamental physicochemical parameter that affects passive diffusion through biological barriers and, as a consequence, bioavailability and biodistribution. However, this parameter is often neglected, and it is quite difficult to find diffusion coefficients of small molecules of pharmaceutical relevance in the literature. The available methods to measure diffusion coefficients of drugs all suffer from limitations that range from poor sensitivity to high selectivity of the measurements or the need for dedicated instrumentation. In this work, a simple but reliable method based on time resolved concentration measurements by UV-visible spectroscopy in an unstirred aqueous environment was developed. This method is based on spectroscopic measurement of the variation of the local concentration of a substance during spontaneous migration of molecules, followed by standard mathematical treatment of the data in order to solve Fick’s law of diffusion. This method is extremely sensitive and results in highly reproducible data. The technique was also employed to verify the influence of the environmental characteristics (i.e. ionic strength and presence of complexing agents) on the diffusivity. The method can be employed in any research laboratory equipped with a standard UV-visible spectrophotometer, and could become a useful and straightforward tool in order to characterize diffusion coefficients in physiological conditions and help to better understand the drug permeability process.

Keywords: Diffusion coefficients, UV-visible spectroscopy, Fick’s laws of diffusion, data fitting, molecular modelling, ion strength
1. Introduction

Diffusion is the spontaneous migration of molecules of a substance from regions of higher chemical activity (high concentration) to regions of lower chemical activity (low concentration) through one or several isotropic environments. This process is of capital importance in pharmaceutics since passive transport of active pharmaceutical ingredients (APIs) remains a significant (and often primary) mechanism of biological membrane permeation (1) and therefore the diffusion process is crucial for reaching an appropriate bioavailability. According to Stokes-Einstein equation (Eq. 1), the diffusivity $D$ ($\text{cm}^2/\text{sec}$) is directly proportional to the absolute temperature $T$ (K) and Boltzmann constant $K_b$ ($\text{cm}^2/\text{g}*\text{s}^2/\text{K}$) but inversely proportional to the viscosity of the media $\eta$ ($\text{g}/\text{cm}*\text{sec}$) and the hydrodynamic radius $r$ (cm) of the molecules.

$$D = \frac{k_b T}{6\pi\eta r} \quad \text{Equation 1}$$

The relation between diffusion and flux of a drug substance in aqueous environment can be described by Fick’s first law as:

$$j = -D \frac{dc}{dx} \quad \text{Equation 2}$$

Where $j$ represents the net flux ($\text{mol}/\text{cm}^2*\text{sec}$) of drug substance through the media, $dc/dx$ represents the concentration gradient and $D$ is the diffusion coefficient. $D$ is an essential parameter to understand pharmaceutically relevant processes such as mass transport through barriers (i.e. absorption) and bioavailability of APIs. However, pharmaceutical research has for years overlooked the importance of this parameter in favor of the measurement of the apparent permeability coefficient ($P_{app}$, 2-4). Since water is the primary component of the human body, a proper quantification of $D$ values for APIs should be of crucial importance for a proper evaluation of the permeability process. Reliable $D$ values, together with a proper evaluation of partition coefficients (LogP), could help to elucidate the role of unstirred water.
layers (UWL) (5) and interface transitions in passive transport through barriers (6). The scientific literature is significantly lacking in diffusivity values for APIs in aqueous environments, and methods to measure them in a simple but reliable manner. Currently, several techniques for measuring diffusion coefficients are available, including holographic laser interferometry (HLI (7)), electron speckle pattern interferometry (ESPI (8)), mass transfer through barrier studies (9), Taylor dispersion analysis (TDA (10,11)), nuclear magnetic resonance (NMR (12)), dual-focus fluorescence correlation spectroscopy (FCA (13)) and fluorescence recovery after photobleaching (FRAP (14)) just to mention the most employed ones. Just a few of these techniques (TDA, NMR and mass transfer studies) have been applied to estimate diffusivity of APIs, the reason being that all the techniques listed above show limitations when facing pharmaceutically relevant problems such as poor sensitivity (e.g. NMR), high specificity (e.g. FRAP) and the need for dedicated instrumentation (e.g. HLI, ESPI, FCA, TDA and NMR which are not standard instrumentations in most pharmaceutical research laboratories).

Another approach to estimate diffusivities of small molecules in aqueous environment is to predict them from molecular properties such as molecular volume, molar volume or by molecular simulation (15). However, all these in silico approaches are prone to uncertainty and do not provide a complete interpretation of diffusion in the case of environmental changes such as ion strength or presence of complexing agents. In this work, a simple, versatile and easily implemented spectroscopic method (16, 17) for diffusion coefficient measurement is introduced. This versatile method is based on the measurement of variation of local concentration of a compound in aqueous media and subsequent temporal resolution of diffusion equation by means of analytical as well as numerical mathematical methods. By comparing different approaches to the solution of Fick’s diffusion equation we show that simple non-linear least-squares fitting is sufficient to extract the diffusion coefficient. The
experiments are performed employing a regular quartz cuvette and a standard UV-visible spectrometer. This method is simple, reliable, reproducible and extremely sensitive. Moreover, it has the significant advantage that it could be employed in any research laboratory equipped with a standard UV-visible spectrophotometer and very small changes related to environmental alterations can be measured.

2. Experimental section

2.1 Materials

Sodium-di-hydrogen-phosphate monohydrate (NaH$_2$PO$_4$·H$_2$O), di-sodium hydrogen-phosphate dodeca-hydrate (Na$_2$HPO$_4$·12H$_2$O), sodium chloride (NaCl), sodium hydroxide (NaOH), hydrochloric acid (HCl, ≥ 37%), caffeine, calcein, chloramphenicol, ketoprofen, nitrofurantoin, tetracycline hydrochloride, trimethoprim, vancomycin, and β-cyclodextrin (βCD) were all purchased from Sigma Aldrich Chemie GmbH (Steinhein, Germany). Paracetamol was purchased from Norsk Medisinaldepot AS (Oslo, Norway). Penicillin G employed was USP Reference Standard (Timbrook, USA). All chemicals were of analytical grade.

2.2. UV-visible localized spectroscopy

2.2.1 Preparation of the solutions

Phosphate buffer saline (PBS) 73 mM was prepared mixing a solution of NaH$_2$PO$_4$·H$_2$O (2.2% W/V) with a solution of Na$_2$HPO$_4$·12H$_2$O (1.8 % W/V) in a ratio 1 to 5. The pH of the PBS was adjusted to 7.3-7.4 (pH meter Lab 744, Metrohm AG, Herisau, Switzerland) by the addition of NaOH solid pellets. Tonicity of the PBS solution was adjusted to 280-290 mOsm (Semi-Micro Osmometer K-7400, Knauer, Berlin, Germany) by the addition of NaCl. Each of the compounds was than adequately weighted and dissolved in the PBS in order to achieve a concentration of approximately 0.5-1.5 mM. The molecular weights of the compounds and the characteristics of the donor solutions (compound concentrations, buffer concentrations,
solution densities, wavelengths of maximal absorption ($\lambda_{\text{MAX}}$) and specific absorptivities ($\varepsilon$)) are reported in Table 1.

**Table 1.** General characteristics and composition of the aqueous solutions used as donor environments. The diffusional medium was composed of plain distilled water (DW, density of 1.0015 g/mL). Each of the compounds was detected at its maximum wavelength of absorption ($\lambda_{\text{MAX}}$) and the local concentration was calculated using its specific absorptivity ($\varepsilon$).

<table>
<thead>
<tr>
<th>Diffusional medium</th>
<th>Compound name</th>
<th>Molecular Weight (g/mol)</th>
<th>Nominal conc. (mM)</th>
<th>Buffer conc. (mM)</th>
<th>Density* (g/mL)</th>
<th>$\lambda_{\text{MAX}}$ (nm)</th>
<th>$\varepsilon$ (cm$^2$/µmol)</th>
</tr>
</thead>
<tbody>
<tr>
<td>DW</td>
<td>Caffeine</td>
<td>194.2</td>
<td>1.23</td>
<td>72.8</td>
<td>1.0086</td>
<td>269</td>
<td>11.3</td>
</tr>
<tr>
<td>DW</td>
<td>Calcein</td>
<td>622.6</td>
<td>1.23</td>
<td>72.8</td>
<td>1.0083</td>
<td>294</td>
<td>11.3</td>
</tr>
<tr>
<td>DW</td>
<td>Chloramphenicol</td>
<td>323.2</td>
<td>1.20</td>
<td>72.8</td>
<td>1.0095</td>
<td>277</td>
<td>10.0</td>
</tr>
<tr>
<td>DW</td>
<td>Ketoprofen</td>
<td>254.3</td>
<td>1.17</td>
<td>72.8</td>
<td>1.0064</td>
<td>257</td>
<td>18.4</td>
</tr>
<tr>
<td>DW</td>
<td>Nitrofurantoin</td>
<td>238.2</td>
<td>0.77</td>
<td>72.8</td>
<td>1.0113</td>
<td>274</td>
<td>13.7</td>
</tr>
<tr>
<td>DW</td>
<td>Paracetamol</td>
<td>151.2</td>
<td>1.42</td>
<td>72.8</td>
<td>1.0079</td>
<td>236</td>
<td>13.2</td>
</tr>
<tr>
<td>DW</td>
<td>Penicillin G</td>
<td>334.4</td>
<td>0.60</td>
<td>72.8</td>
<td>1.0071</td>
<td>207</td>
<td>19.9</td>
</tr>
<tr>
<td>DW</td>
<td>Tetracycline</td>
<td>444.4</td>
<td>0.83</td>
<td>72.8</td>
<td>1.0197</td>
<td>361</td>
<td>14.8</td>
</tr>
<tr>
<td>DW</td>
<td>Trimethoprim</td>
<td>290.3</td>
<td>1.19</td>
<td>72.8</td>
<td>1.0085</td>
<td>278</td>
<td>5.6</td>
</tr>
<tr>
<td>DW</td>
<td>Vancomycin</td>
<td>1449.3</td>
<td>1.20</td>
<td>72.8</td>
<td>1.0011</td>
<td>280</td>
<td>6.7</td>
</tr>
</tbody>
</table>

*Data are reported as mean of three measurements. SD below 0.5%

Solutions of βCD (used in the diffusion experiments to investigate complexation effects) were prepared by adding solid powder of the dextrin derivative (3.4-4 mg) directly into 2 mL of ketoprofen neutral and acidic solution (pH 1.0, obtained by mixing 1.9 mL of neutral ketoprofen solution plus 0.1 mL of HCl) in order to achieve a molar ratio drug:βCD of approximately 1:1.

2.2.2 Experimental procedure

For the spectrophotometric measurements, a double array VWR (VWR International, Radnor, USA) UV-visible spectrophotometer (model UV-6300 PC) equipped with a Hellma® Suprasil® (Sigma-Aldrich) quartz absorption cuvettes (chamber volume of 700 µL and path length of 10 mm) was employed. The experimental setup is schematized in Fig. 1.
Both reference and sample cuvette were filled with 675 µl of distilled water and placed in the respective compartment of the spectrophotometer. At time (t) = 0 sec (starting of the experiment), 25 µL of drug solution was gently injected in the bottom of the sample cuvette by a needle syringe. The reference and sample cuvette were both sealed with parafilm in order to avoid evaporation of the solvent. Absorbance readings were recorded at fixed wavelength (Table 1) at regular time intervals (120 sec) for 24 hours at room temperature (23-24°C).

Absorbance was recorded at a specific position \( x_1 \) corresponding to 0.51 cm from the bottom of the cuvette (Figure 2). This was obtained by keeping the sample cuvette lifted 0.60 cm (Hard Head calliper, Jula Norge AS, Lørenskog, Norway) from the bottom of the cuvette holder.
Figure 2. Adaptation of the mono-dimensional diffusional model used to the cuvette geometry and related relevant spatial points (initial point $x_0$, measurement points $x_1$ and $x_2$ and total length of the diffusion chamber L).

For each of the API studied, two independent experiments were conducted (n=2) and the final diffusional profile (employed for the mathematical data treatment and diffusivity calculations) was obtained by averaging the respective absorbances at each time point. The average variance (in percentage, %) between the absorbance registered at each time point between the two parallel experiments was used to estimate the experimental error ($\leq 5\%$).

2.2.3 Mathematical model and UV-visible spectroscopy data interpretation

The mathematical approach used in this work to fit the experimental data obtained by UV-visible spectroscopy to calculate the diffusion coefficients was based on the standard diffusion equation (Eq. 3) and well-known classical physical models (9, 18). Due to the geometry of the experimental setup (Figure 2), the diffusion process was approximated with a one-dimensional model. The diffusion equation (Eq. 3) describes the concentration of a solute over time and space in a homogenous medium (assuming constant diffusivity) as:

\[
\frac{\partial C}{\partial t} = D \frac{\partial^2 C}{\partial x^2}
\]
Where $c$ represents the concentration of the substance, $t$ the time, $x$ the position and $D$ the diffusivity. In theory, any $x$ between 0.51 cm and 1.11 cm can be selected (Fig. 2) by lifting the cuvette from its standard recording position. In this work, the recording point was primarily set to 0.51 cm (see experimental procedure section).

The first mathematical approach used to find numerical solution of $D$ was analytical. This approach is based on the assumption that for times $t$, $t < \frac{L^2}{D}$ and $x < \frac{L}{D}$ (where $L$ is 3.30 cm, Fig. 2) the container can be considered to be infinite in length and the differential equation can be solved via its Fourier transform.

Based on the governing equation (Eq. 3) and the given boundary conditions (See Supporting Information A), the following analytical solution was derived (Eq. 4, see Supporting Information A for a detailed description of this equation):

\[
\frac{\partial c(x, t)}{\partial t} = D \frac{\partial^2 c(x, t)}{\partial x^2} \quad \text{Equation } 3
\]

\[
c(x, t) = \frac{A}{\sqrt{\pi}} \frac{e^{-\frac{x^2}{4Dt}}}{\sqrt{2\sigma^2 + 4Dt}} \quad \text{Equation } 4
\]

In this equation, $\sigma$ represents the width of the initial distribution and it is used as a fitting parameter. Equation 4 was fitted to the experimental data (concentration over time profiles) using a non-linear fitting program in order to find diffusivity values for each API investigated. An alternative approach used was to use a numerical approach to find diffusivity values. In this case, the initial concentration distribution was approximated by a standard Gaussian distribution centered in the origin as (Fig.3, (18)):

\[
c(x, 0) = \alpha e^{-\frac{x^2}{2\sigma^2}} \quad \text{Equation } 5
\]

Where $\sigma$ is the full width at half maximum and $\alpha$ the maximum of the initial Gaussian distribution obtained by the equilibrium concentration ($c_f$ i.e. the final equilibrium concentration of the compound injected into the cuvette homogeneously diffused) and the
length of the diffusional pathway (L) (see Supporting Information B for a detailed description of the calculation of these parameters). Since there was no flux into or out of the cuvette (conditions of mass preservation), the boundary conditions at \(x_0\) (0.00 cm) and \(L\) (3.30 cm), (Fig. 2) were (Eq. 6a and 6b):

\[
\frac{\partial c(x, t)}{\partial x} = 0 \quad (a) \quad \frac{\partial c(L, t)}{\partial x} = 0 \quad (b) \quad \text{Equation 6}
\]

The diffusion equation (Eq. 3) with the initial condition (Eq. 5) and the boundary conditions (Eq. 6) has been solved numerically with a finite differences scheme (19) (for additional information see Supporting Information B). To estimate the parameters \(D\) and \(\sigma\) (variable parameters) a global optimization method based on simulated annealing was used (Matlab\(^\text{®}\) program from Mathworks Inc., Natick, USA).

**Figure 3.** Example of concentration vs position (assuming diffusion over one-dimension).

The concentration distribution is initially \((t_0)\) represented by a Gaussian curve (blue line) centered on the origin. The red and the yellow lines respectively represent the concentration distribution after a short time \((t_1)\) and a longer time \((t_2)\). The black-dotted lines represent the two different observation points \((x_1\) and \(x_2\)).

Diffusion experiments of caffeine were used in order to calibrate \(x\) in the experimental set-up. Specifically, two diffusion experiments were conducted at recording at two different measurement points: \(x_1\) (0.51 cm) and \(x_2\) (1.11 cm, measured by caliper). In each of the calibration experiments, recording position \((x_1\) or, alternatively, \(x_2\)) were let to optimization.
The best fitting was obtained with x values of 0.51 and 1.11 cm respectively, in good agreement with direct caliper measurement.

2.3 Estimation of diffusion coefficients from molecular modelling

Diffusion coefficients of the APIs were estimated from molecular properties using two different approaches. The first was to calculate the molecular radii from the solvent-accessible molecular volumes (SAMV) of the APIs, which were determined using the QikProp program (QikProp, Schrödinger, LLC, New York, NY, 2017).

The second approach was to use the molar volume, which was calculated employing the ChemSketch software (Advanced Chemistry Development Inc., Toronto, Canada). In both cases the API molecules were approximated to be spherical and the calculated radii (Table 2) were used to estimate the diffusion coefficients employing Stokes-Einstein equation (Eq. 1).

Table 2. Molecular volumes and solvent-accessible molecular volumes (SAMV) estimated by molecular modelling and the respective calculated molecular radii.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Molar volume$^a$ cm$^3$/mol</th>
<th>Molecular radii estimated from molar volume Å</th>
<th>SAMV$^b$ Å$^3$</th>
<th>Molecular radii estimated from SAMV Å</th>
</tr>
</thead>
<tbody>
<tr>
<td>Caffeine</td>
<td>133.3</td>
<td>3.7</td>
<td>646.9</td>
<td>5.4</td>
</tr>
<tr>
<td>Calcein</td>
<td>378.6</td>
<td>5.3</td>
<td>1611.0</td>
<td>7.3</td>
</tr>
<tr>
<td>Chloramphenicol</td>
<td>208.8</td>
<td>4.4</td>
<td>881.4</td>
<td>5.9</td>
</tr>
<tr>
<td>Ketoprofen</td>
<td>212.2</td>
<td>4.4</td>
<td>865.7</td>
<td>5.9</td>
</tr>
<tr>
<td>Nitrofurantoin</td>
<td>163.5</td>
<td>4.0</td>
<td>766.0</td>
<td>5.7</td>
</tr>
<tr>
<td>Paracetamol</td>
<td>120.9</td>
<td>3.6</td>
<td>567.8</td>
<td>5.1</td>
</tr>
<tr>
<td>Penicillin G</td>
<td>235.1</td>
<td>4.5</td>
<td>1044.2</td>
<td>6.3</td>
</tr>
<tr>
<td>Tetracycline</td>
<td>273.1</td>
<td>4.8</td>
<td>1203.9</td>
<td>6.6</td>
</tr>
<tr>
<td>Trimethoprim</td>
<td>231.8</td>
<td>4.5</td>
<td>928.7</td>
<td>6.1</td>
</tr>
<tr>
<td>Vancomycin</td>
<td>912.3</td>
<td>7.1</td>
<td>3687.7</td>
<td>10.0</td>
</tr>
</tbody>
</table>

$^a$ Calculated with ChemSketch software (Advanced Chemistry Development Inc.)

$^b$ Calculated with QikProp program (QikProp, Schrödinger, LLC)

3. Results and discussion

3.1 Validation experiment with caffeine
One of the biggest challenges to overcome in the proposed diffusional model (mimicking the UWL) was to avoid turbulence during injection of the donor solution to the diffusion media (Fig. 2). To solve this problem, a very simple but effective expedient based on density gradient was used. In Table 1 the general properties of all solutions used as donor media and the diffusion media are reported. The presence of buffering salts and compounds in solution (Table 1) resulted in a slightly higher density for the donor aqueous environment compared to the diffusion media (just plain distilled water). This density difference allowed injection/deposition of the donor solution in the bottom of the cuvette without any turbulence or movement of liquids that could hamper proper measurement of diffusion (as represented in Fig. 2). It should be underlined that both solutions (donor and diffusing media) were primarily composed of water so there was no interphase between them (isotropic media, one-phase compartment). In Fig. 4, the concentration vs time curve obtained for caffeine at $x_1$ is reported as an example, but this could be generalized to all investigated compounds. As can be seen in Fig. 4, the concentration of the drug increased locally to a maximum value ($0.14 \, \mu \text{mol/cm}^3$) and then the concentration slowly decreased until reaching equilibrium (not reached in 24 hours, period of the experiments). The best fit obtained with simulated annealing (Fig. 4, blue-dashed line) was $x = 0.51$ cm, confirming the caliper measurement.
Figure 4. Variation of local concentration of caffeine over time measured at $x_1$ (0.51 cm). The blue-dotted line represents the experimental data whereas the red-dashed line represents the best fit (numerical approach).

To verify that the diffusion process measured by UV-visible spectroscopy was reliable and that the diffusional model designed was appropriate, a second data set was recorded for caffeine at $x_2$ (1.11 cm) using the previously detected diffusion coefficient (at $x_1 = 0.51$ cm) as a constant and setting $x$ as variable. In Fig. 5, the variation of the concentration of caffeine recorded at $x_2$ over time is reported. As expected, the increase of the concentration over time was slower (highest concentration approx. 0.06 µmol/cm$^3$) due to the observation point being further from $x_0$.

Figure 5. Variation of local concentration of caffeine over time measured at $x_2$ (1.11 cm). The blue-dotted line represents the experimental data whereas the red-dashed line represents the best fit obtained with the simulated annealing ($D = 9.4 \times 10^{-6}$ cm$^2$/sec).

Interestingly, the data fitting of the curve obtained by simulated annealing (red-dashed line) mimicked closely the behavior observed experimentally (blue-dotted line) also in this case. It should be noted that, even though in this experiment $D$ was used as constant and $x$ let to optimization, the $x$ found was in very good agreement with the caliper measured (1.11 cm) certifying the validity of the experimental set-up for diffusion studies developed. We also
investigated an analytical-based method employing the value of 0.51 cm as fixed parameter for the measurement position \( x_1 \).

3.2 Experimental diffusion measurements

In Table 3, the diffusion coefficients calculated with the numerical and analytical approaches are reported together with the data obtained from the literature (when applicable) for the compounds investigated.

Table 3. Diffusion coefficients obtained experimentally by the UV-visible spectroscopic method and subsequent analytical and numerical data treatment (Fitting error below 1 %). When applicable, results are compared with available literature data.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Analytical approach ( D ) ((10^{-6} \text{ cm}^2/\text{sec}))</th>
<th>Numerical approach ( D ) ((10^{-6} \text{ cm}^2/\text{sec}))</th>
<th>Literature values ( D ) ((10^{-6} \text{ cm}^2/\text{sec}))</th>
</tr>
</thead>
<tbody>
<tr>
<td>Caffeine</td>
<td>9.0</td>
<td>9.4</td>
<td>7.8 (^{20}), 6.2 (^{21})</td>
</tr>
<tr>
<td>Calcein</td>
<td>3.8</td>
<td>3.8</td>
<td>3.3 (^{22})</td>
</tr>
<tr>
<td>Chloramphenicol</td>
<td>6.6</td>
<td>6.3</td>
<td>5.7 (^{23})</td>
</tr>
<tr>
<td>Ketoprofen</td>
<td>6.4</td>
<td>6.8</td>
<td>-</td>
</tr>
<tr>
<td>Nitrofurantoin</td>
<td>7.1</td>
<td>7.0</td>
<td>-</td>
</tr>
<tr>
<td>Paracetamol</td>
<td>7.8</td>
<td>7.7</td>
<td>6.6 (^{24})</td>
</tr>
<tr>
<td>Penicillin G</td>
<td>6.5</td>
<td>6.4</td>
<td>4.0 (^{25})</td>
</tr>
<tr>
<td>Tetracycline</td>
<td>5.8</td>
<td>5.9</td>
<td>-</td>
</tr>
<tr>
<td>Trimethoprim</td>
<td>5.6</td>
<td>5.6</td>
<td>-</td>
</tr>
<tr>
<td>Vancomycin</td>
<td>2.9</td>
<td>3.0</td>
<td>-</td>
</tr>
</tbody>
</table>

Data recorded at \( x_1 \) position \((0.51 \text{ cm})\)

As shown in Table 3, the diffusivities obtained with the two different mathematical approaches are quite similar, with a variation between 0% (calcein, trimethoprim) and 6% (for ketoprofen). This justifies the thought that both mathematical approaches are equally good for solving local concentration profiles in such geometrical diffusional parameters (i.e. size of a cuvette). From these results, it appears that the analytical approach works well with the geometry of a standard quartz cuvette and small to medium-sized molecules. However, it
cannot be excluded that this assumption might not be valid anymore for smaller molecules (with higher D); however such scenarios were not investigated in this work. In cases where diffusivities might be affected by specific parameters (e.g. high viscosity, strong electrostatic field) also the numerical approach should be adjusted, with the application of more complex equation. However, for the experimental conditions employed in this work, the standard diffusion equation resulted adequate (fitting error below 1%). Moreover, the experimental data results are quite comparable with the available literature data ((20-25) linear fitting $R^2$ of 0.8). Due to the limited data available in the literature it is difficult to clearly state if our method is more accurate than others. It should be noted that the D values obtained in this work are generally higher than the ones reported in the literature. Interestingly, using caffeine literature value ($7.8*10^{-6}$ cm$^2$/sec) to re-scale our data, our values became identical to the literature values for calcein, chloramphenicol and paracetamol. A discrepancy still exist for penicillin G (rescaled value obtained $5.7 *10^{-6}$ cm$^2$/sec), but the difference is much smaller. However, the literature data (25) refers to an experiment performed with potassium penicillin in plane water therefore the discrepancy is most probably due to the different experimental conditions (i.e. pH, tonicity (26)) used. These data suggest that the D values we identified should therefore be considered “relative” but, using a standard, the spectroscopic method described in this work results suitable in order to measure absolute diffusivities.

3.3 Comparison with model predictions

In Figure 6 the experimentally determined diffusion coefficients (analytical approach) are compared with the diffusivities calculated by two different $in silico$ parameters (solvent-accessible molecular volume (SAMV) and molar volume).
Figure 6. Correlation between the diffusivity constants measured experimentally (and subsequent analytical data treatment) and estimated by molecular properties (solvent-accessible molecular volume (SAMV) and molar volume). Calculated D error was below 1%.

As it can be seen, the correlation between experimentally determined and calculated diffusivities is quite linear ($R^2 = 0.9$), independently of the molecular descriptor used to estimate diffusivity. D values measured in this work result generally slightly higher than the one obtained by in silico prediction (Fig. 6). One of the reasons might be that we measure the diffusivity of the chromophore which can be higher than the diffusivity of the center of mass.

The diffusivities measured via the UV-technique should thus be consider “relative” diffusivities and not absolute values. However, the method disclosed in this work can be considered extremely reliable and can reliably monitor even very small changes in diffusivity.

3.4 Effect of ion strength and complexation on diffusivity
In order to explore the potentials of this technique to solve relevant pharmaceutical issues, we applied this technique to investigate whether it was possible to measure the influence of ion strength and the presence of βCD on the diffusion process. First, we tried to verify if the alteration of the environment of diffusion could affect the diffusion constants. In the first experiments we wanted to verify if the ion strength (i.e. tonicity) could influence the diffusion constant of caffeine. As it can be seen from Table 4, reducing the tonicity of donor solution did not significantly alter the diffusivity of caffeine. On the contrary, the diffusivity of caffeine was highly increased (25 % increase) by the increased ionic strength (i.e. tonicity).

**Table 4.** Influence of environmental ion strength on the diffusion coefficients of caffeine.

Hypertonic solution was obtained by the addition of NaCl.

<table>
<thead>
<tr>
<th>Caffeine conc. (μmol/cm³)</th>
<th>pH</th>
<th>Ionic strength (mOsm)</th>
<th>Diffusivity (10⁻⁶ cm²/sec)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Isotonic media</td>
<td>1.23</td>
<td>7.4</td>
<td>290</td>
</tr>
<tr>
<td>Hypotonic media</td>
<td>1.42</td>
<td>7.6</td>
<td>35</td>
</tr>
<tr>
<td>Hypertonic media</td>
<td>1.34</td>
<td>7.2</td>
<td>521</td>
</tr>
</tbody>
</table>

*Single experiment

This phenomenon can be explained by the fact that at higher concentration of ions, electrical fields and osmotic gradient may start to play a stronger role in the passive diffusion of APIs (26), synergistically promoting passive diffusion of the API molecules. In the second experiment we wanted to verify the effect of complexing agents on passive diffusion. For this purpose, a solution of ketoprofen and βCD was prepared at pH 1 and used as donor solution. At acidic pH, ketoprofen in solution is primarily in its unionized form (pKa 4.7) and therefore it expresses the highest affinity for the dextrin rings (27). In fact, the equilibrium constant of the ketoprofen-βCD complex is approximately 20 % higher in acidic...
pH (unionic ketoprofen) than in neutral/basic pH (ionic ketoprofen) (27). Using data fitting and simulated annealing it was possible to calculate a new diffusion coefficient of $5.04 \times 10^{-6}$ cm$^2$/sec for ketoprofen in the presence of βCD that corresponded to a 25% reduction in comparison to ketoprofen alone. This discrepancy was also confirmed by applying analytical mathematical interpretation of the data. In order to verify if the reduction of D could be related to other factors rather than complexation (e.g. viscosity increment given by the presence of βCD) a diffusion experiment of ketoprofen in the presence of βCD was also conducted as reference at pH 7.4. At this pH ketoprofen molecules are expected to be primarily ionized, therefore having reduced affinity for βCD (i.e. ketoprofen molecules should be primarily uncomplexed). In this case the reduction in D value of ketoprofen was just 7% (analytical solution). This result clearly shows that the presence of complexing agents has a significant effect on the diffusivity of ketoprofen, and that the magnitude of this effect is directly related to the drug-βCD equilibrium constant (pH dependent).

4. Conclusion

In this work, a simple and versatile technique based on temporal resolution of concentration measurement by UV-visible spectroscopy for the determination of API diffusion coefficients in unstirred aqueous environment has been successfully developed. This technique is simple, of high sensitivity and it could be potentially applied to almost all drugs as well as to new chemical entities. In this work the technique has been successfully employed to verify the influence of environmental characteristics on diffusion. This technique might become an important tool to gain a better understanding of relevant biopharmaceutical problems such as permeability of drugs through biological barriers and comprehension of the effect of the diffusional environment on diffusion.

Acknowledgments
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Supporting information

A. Detailed description of the analytical solution of the diffusion equation.

B. Detailed description of the numerical approach employed.
References


Figure captions

Figure 1. Schematic representation of localized UV-visible spectroscopy experimental setup.

Figure 2. Adaptation of the mono-dimensional diffusional model used to the cuvette geometry and related relevant spatial points (initial point $x_0$, measurement points $x_1$ and $x_2$ and total length of the diffusion chamber $L$).

Figure 3. Example of concentration vs position (assuming diffusion over one-dimension).

Figure 4. Variation of local concentration of caffeine over time measured at $x_1$ (0.51 cm). The blue-dotted line represents the experimental data whereas the red-dashed line represents the best fit (numerical approach).

Figure 5. Variation of local concentration of caffeine over time measured at $x_2$ (1.11 cm). The blue-dotted line represents the experimental data whereas the red-dashed line represents the best fit obtained with the simulated annealing ($D = 9.4 \cdot 10^{-6}$ cm$^2$/sec).

Figure 6. Correlation between the diffusivity constants measured experimentally (and subsequent analytical data treatment) and estimated by molecular properties (solvent-accessible molecular volume (SAMV) and molar volume). Calculated $D$ error was below 1%.