magnetochemistry

MDPI

Article

Low Field NMR Determination of p*Ka* Values for Hydrophilic Drugs for Students in Medicinal Chemistry

Aleksandra Zivkovic [1], Jan Josef Bandolik [1], Alexander Jan Skerhut [1], Christina Coesfeld [1], Miomir Raos [2], Nenad Zivkovic [2], Vlastimir Nikolic [3] and Holger Stark [1,*]

1 Institute of Pharmaceutical and Medicinal Chemistry, Heinrich Heine University Düsseldorf, Universitätsstr. 1, 40225 Duesseldorf, Germany; aleksandra.zivkovic@hhu.de (A.Z.); Jan.Bandolik@uni-duesseldorf.de (J.J.B.); alexander.skerhut@uni-duesseldorf.de (A.J.S.); christina-coesfeld@gmx.de (C.C.); stark@hhu.de (H.S.)

2 Faculty of Occupational Safety, University of Nis, Carnojevica 10A, 18000 Nis, Serbia; miomir.raos@znrfak.ni.ac.rs (M.R.); nenad.zivkovic@znrfak.ni.ac.rs (N.Z.)

3 Faculty of Mechanical Engineering, University of Nis, Aleksandra Medvedeva 14, 18000 Nis, Serbia; vnikolic@masfak.ni.ac.rs

* Correspondence: stark@hhu.de; Tel.: +49-211-811-0478

Received: 6 September 2017; Accepted: 19 September 2017; Published: 20 September 2017

Abstract: For an interdisciplinary approach on different topics of medicinal and analytical chemistry, we applied a known experimental p*Ka* value determination method on the field of the bench top nuclear magnetic resonance (NMR) spectrometry of some known biologically active pyridine-based drugs, i.e., pyridoxine hydrochloride, isoniazid, and nicotine amide. The chemical shifts of the aromatic ring protons in the ^{1}H NMR spectrum change depending on the protonation status. The data were analyzed on dependence of the chemical shifts by different pH (pD) environments and then the p*Ka* values were calculated. The p*Ka* values obtained were in agreement with the literature data for the compounds, searched by the students on web programs available at our university. The importance of the p*Ka* values in protein-ligand interactions and distribution etc. of drugs was brought up to the students' attention. In addition, by the use of a free web application for p*Ka* values prediction, students calculated the predicted modeled p*Ka* value. The experimental and in-silico approaches enhance the tool box for undergraduate students in medicinal chemistry.

Keywords: p*K*a value; low field NMR; molecular properties; pharmacokinetics; undergraduate pharmacy students

1. Introduction

The protonation status of drugs under physiological and pathophysiological conditions is of mandatory importance for the understanding of drug action concerning pharmacodynamics and the pharmacokinetic mechanism of action. NMR experiments deliver a versatile experimental method for the determination of p*Ka* values and thereby increase the knowledge on NMR techniques. Due to the complex theoretical background, it has remained a challenge to teach the basics in NMR spectroscopy. Although this analytical technique has been one of the most important techniques for structure identification for decades, it has been difficult to teach this as practical course containing students' experimental NMR spectroscopy because of the price of the instruments and the handling. The development of bench-top NMR spectrometers has made this kind of teaching possible [1–3]. Interest for complex and high molecular weight protein structures has been an inspiration for the amazing developments in the high-resolution nuclear magnetic resonance. This kind of structural

analysis is usually performed in the strong magnetic field generated up to 1.2 GHz by huge magnets. The technological progress allowed the development of the smaller but more powerful superconducting magnets. For many reasons, the student experiments done in instrumental analytic courses on sophisticated equipment were mostly limited to spectra interpretation. There is still a strong need to teach undergraduate students this attractive technique through experimentation.

The NMR improvement in strength, size, homogeneity, and temperature stability endorsed the production of small, portable NMRs. There are a few low-field spectrometers available on the market today. There is no need to use the cryogens, and there are no additional maintenance costs [4]. There are other experiments developed for the student lab involving hydrogen bond dynamics determination, kinetic determination, oil spill determinations, quantitative determination, etc. In order to introduce our students to ^{1}H NMR spectroscopy, we offer the possibility to solve a physico-chemical problem (determination of p*Ka* values of drugs) employing the newly obtained knowledge on NMR spectroscopy [5–8]. We focused on developing the experiments that stress the importance of the NMR techniques in pharmaceutical analytics and drug design. We previously published two experiments that deepened the understanding of structure determination [2] and quantification [1] using NMR spectroscopy. Generally, NMR spectroscopy in a student lab is regarded as a technique for structural determination. It is rarely considered as reliable method for experimental p*Ka* value determination, although the method is common in science, especially in protein determination [9]. However, with the full set of experiments, we are trying to show our students how NMR can be also employed for determination of physiochemical characteristics of the compounds. The significance of acid/base properties in drug discovery and pharmacokinetics research is of the great importance in medicinal chemistry. The p*Ka* value of the drug influences drug lipophilicity, solubility, protein binding, and permeability. All these affect pharmacokinetic characteristics of the drugs, such as adsorption, distribution, metabolism, and excretion (ADME) [10,11]. Furthermore, the p*Ka* value and acidic/basic characteristics can help us determine if the drug is suitable for oral application or not. The determination of those characteristics early in the drug development process allows pharmaceutical companies to reduce the number of failed studies. It is common knowledge that the pH of different body compartments is very different, varying from roughly pH of 1 to pH of 9. Depending on the p*Ka* value of the drug, an optimal place for drug absorption can be predicted [12].

We designed an experiment for undergraduates with simple drug structures but tried to make our students familiar with the possibility that the same approach can also be applied on more complex structures. To foster research conditions, our goal is that students search for the drugs and the acidity/basicity of the drugs and model the data on their own using web applications. The assistant's role is to encourage the students to research and ask questions about the method and the results and to discuss the conclusions with the students. The main learning goals are (i) to deepen the understanding of the ^{1}H NMR technique, (ii) to understand the connection in-between the chemical characteristics and chemical shifts, (iii) to understand the effects that p*Ka* value has on pharmacokinetics, and (iv) to process the data on their own using MNOVA Software.

We applied Peer-Led Team Learning (PLTL), benefiting the student collaboration. We supplied the group leaders with the publications determining the p*Ka* values of the solvents and let them form groups of three to discuss the approach and understand how it works [9]. Afterward, the students were asked to cross-reference the Pharmacopoea Europaea (Ph. Eur.) (searching for drugs containing a pyridine substructure) and find compounds that could potentially be suitable for determination using this approach. After cross-referencing their list with the drug inventory list we supplied, they decided on their own which compound they wanted to use for the determination. The work of the students was facilitated with pre-leaders and instructors. The small group learning setting is very beneficial [13].

The pH-dependent change in chemical shift can be followed by ^{1}H NMR measurements and used for p*Ka* value determination. For reasons of comparison, we took advantage of the chemical and therefore magnetic changes due to protonation of the pyridine ring on related drugs (Figure 1). We determined the p*Ka* values, corresponding the nitrogen in the heterocyclic pyridine ring in

isoniazid, pyridoxine hydrochloride, and nicotinamide (Figure 2). The NMR-based experiments on the physicochemical properties of known drugs enables the students to combine the protonation status to ^{1}H NMR spectra interpretation (*cf*. Supporting Information).

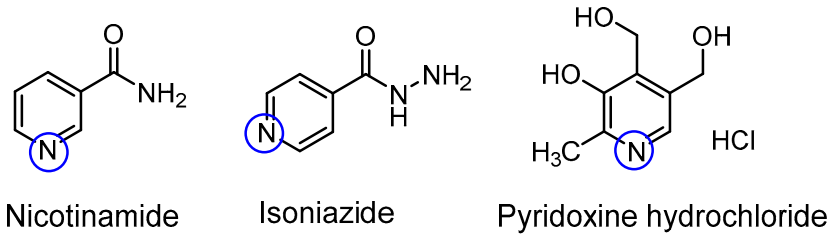

Figure 1. p*Ka* values of the nicotinamide, isoniazid, and pyridoxine hydrochloride were determined.

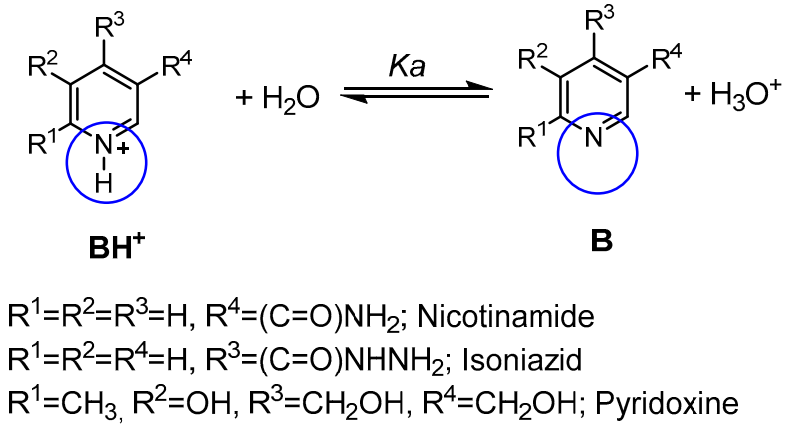

Figure 2. Protonation changes of the drugs in water.

2. Results

2.1. Method Adoption

In a previously published experiment (developed in 1973 and adopted in 2012 [9,14]), pyridine, picoline, and lutidine solvents were used for the determination. However, for our approach, it was important to show students that simple, basic scientific methods can be transferred and applied to the more complex structures they focus on in their studies, as the pharmacokinetics of drugs is one of the most important issues in drug development. In the classic high field NMR machines, a deuterated solvent with internal standard is used, whereas our instrument uses an external lock and can work in non-deuterated solvents and without internal standards. The method itself was only slightly modified. The internal standard was omitted, whereas the pH was adjusted in a related manner as described in ref. [9]. These procedures have been applied to water-soluble pyridine-based drugs (Figure 1, *cf*. Supplementary Materials Figures S1–S7, Tables S1–S3).

We observed the following protonation changes of the drugs in water:

The ionization constant for the equilibrium is given in the classic Equation (1).

$$Ka = \frac{[H_3O^+] \quad [B]}{[BH^+]} \tag{1}$$

By transformation into a logarithmic function, Equation (1) is reformed into the Equation (2) [9].

$$pKa = pH + \log \frac{[BH^+]}{[B]} \tag{2}$$

The proton chemical shifts of the cationic and charged molecules are very different. The main reasons for this difference are anisotropy and electron density. When the proton dissociation is faster than the NMR time scale, there is only one signal to be observed representing the average position of the signal, defined by the Equation (3) [9].

$$\delta_{obs.} = \delta_{BH^+} P_{BH^+} + \delta_B P_B \quad (3)$$

Equation (3) can be further simplified, as previously reported in detail in [9] (here shown with the Equations (4)–(6)). This is used to connect the NMR experiment with this physicochemical experiment.

$$p_{B\cdot} = \frac{\delta_{pH=1} - \delta}{\delta_{pH=1} - \delta_{pH=13}} \quad (4)$$

$$p_{B\cdot} + p_{BH^+} = 1 \quad (5)$$

$$pKa = pH + \log \frac{p_{BH^+}}{p_B} \quad (6)$$

For our measurements, we used the mixture of the D_2O and H_2O solutions at a startiing concentration of approximately 20 mg/mL. We measured so called pH* by directly reading the D_2O-solution of the water-calibrated pH-meter. The conversion of the pH* into the pD is done by adding a constant of 0.4. The p*Ka* (H) values were calculated with Equation (7) from a determined p*Ka* (D) [15].

$$pKa\ (D) - pKa\ (H) = 0.076pK\ (H) - 0.05 \quad (7)$$

2.2. Student Experimental Results

This experiment was done by approximately 70 students attending an instrumental analytics course for pharmacists, divided into three-membered groups (*cf.* Supporting Information). As an example, we showed the students a data plot of the pyridoxine NMR titration. Each of aromatic protons can be used for the processing of NMR data to determine the p*Ka* value. Enlarging the region with the protons of interest was helpful when analyzing data (Figure 3).

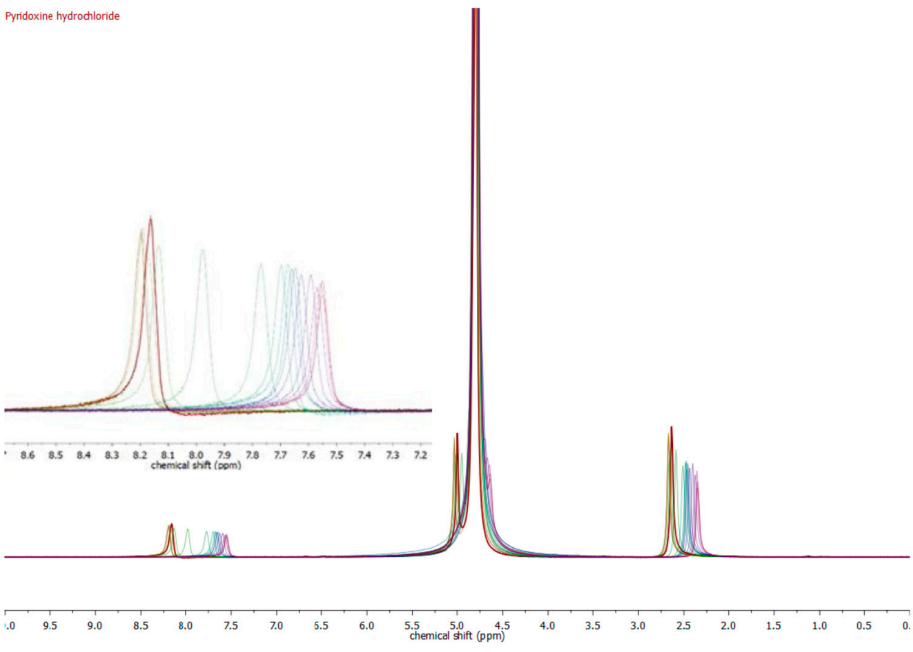

Figure 3. Stacked ^{1}H NMR spectra of pyridoxine at different pH values (starting conc. 20 mg/mL) showing full-scale and enlarged region of interest.

The collected ^{1}H NMR data were processed and plotted against the pD values (i.e., value related to pH in D_2O) (Figure 4). The curves obtained can be compared to regular titration curves. If so, the p*Ka* can be determined as the equivalence point by titration, as determined using a concentrated arc method (Tubbs' method, [16]) or as the first derivation of the function (Excel). Therefore, the determined point can be recalculated using Equation (7) (*cf.* Supporting Information Chart S1). Working with undergraduate students applying of graphical method has advantages as they usually do not have a lot of experience in creating charts. The pH* is measured with a glass electrode upon the addition of either the KOH-solution or the HCl-solution. All experimental details and data can be found in the supporting information.

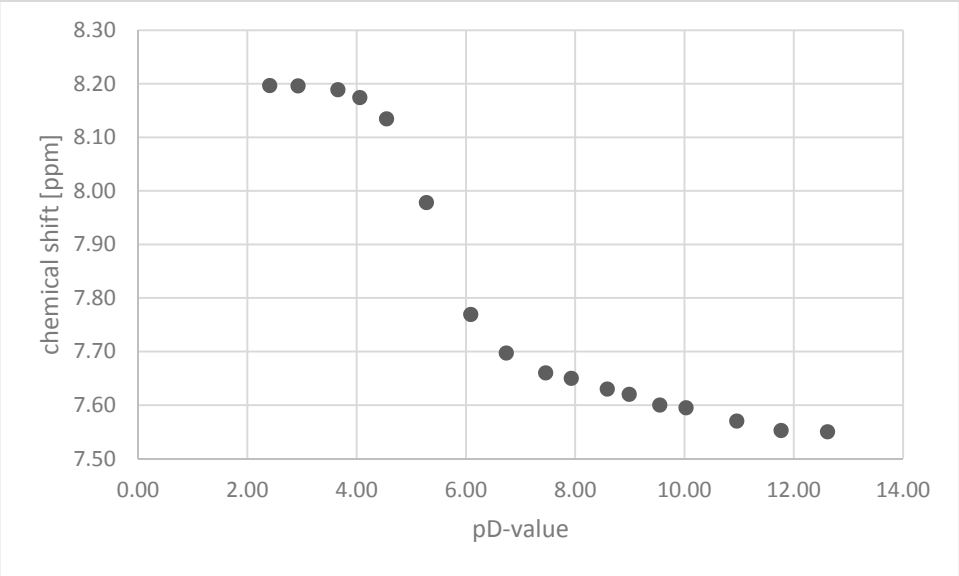

Figure 4. An example plot of the ^{1}H NMR chemical shift of a ring proton of pyridoxine hydrochloride as a function of pD.

The results obtained by students, the corresponding literature values, and the values obtained by the computational approaches are shown in Table 1.

Table 1. The determined p*Ka* values by NMR experiments, literature data, and predicted values based on computational approaches.

Compound	p*Ka* (NMR Method) [b]	p*Ka* [c] (Literature)	p*Ka* (Web-Based Calculation) [d]
Nicotinamide	3.54 ± 0.02	3.35 [17]	3.60
Isoniazid	3.65 ± 0.19	3.50 [18]	3.20
Pyridoxine [a]	5.24 ± 0.15	5.20 [19]	5.60

All p*Ka* values correspond to the p*Ka* values of the heterocyclic ring nitrogen. [a] Used as hydrochloride salt. [b] The student data are the mean value of two determinations and recalculation according to Equation (7). Values for one standard deviation are also included (upper t probability t of 0.10); [c] Ref. given for each value; [d] Calculated using CE and JChem acidity and basicity calculator, https://epoch.uky.edu/ace/public/pKa.jsp.

3. Discussion

With the positive feedback from the students, we evaluated the learning outcome. Working in groups with defined group leader was successful, and the discussions were fruitful. Each group was able to apply the concept and successfully determine the p*Ka* value of interest, which confirms that the methodology is simple enough to be done by students. The applied methodology allowed the students to learn important features of the NMR spectroscopy on their own and employ their learning

with better success than when solving imagined problems in a theoretical course. Hands-on learning in small, peer-lead groups was a new experience for our students. Even though the process was guided by assistants due to the cost issue (list of matches is limited), this gave them an important feeling of working in a scientific setting. Searching through the literature on their own and using even a very simple modeling assignment showed them the possibilities and issues in the science. Stressing the importance of the physicochemical characteristics in the process of drug discovery and in adsorption/distribution/metabolism/elimination (ADME) gave our students a better perspective on how the learning of elemental basics (acidity/basicity, p*Ka* values) is of use with regard to those complex topics. In the final discussion, the importance of the p*Ka* values of drugs in pharmacodynamics and pharmacokinetic mechanisms is generally discussed.

In addition, in a written exam, the students obtained in average 15% more points in the NMR assignments and in electrochemistry assignments involving potentiometric titrations. When asked what were the two most positive aspects of this experiment, the student answered that the most attractive elements for them were to getter on their own the information from the different sources and then to apply those in the experiment and to measure the NMR spectra on their own. To conclude, the modern PLTL-method, combined with molecular modeling and a hands-on NMR experience, had a very positive influence on the knowledge and motivation of the students in the lab.

4. Materials and Methods

4.1. Materials and Instrumentation

A variety of low field instruments are on the market and are suitable for use in this experiment. We used Magritek Spinsolve NMR Benchtop (Magritek, Aachen, Germany) (42.5 MHz) with standard 5 mm NMR-tubes. The measurement used was a 1D PROTON Powerscan measurement using a single 90-degree excitation pulse. The standard 1D measurement used 90-degree excitation pulse as this maximizes the signal in the *x*,*y*-plane where the signal is detected [20].

4.2. Prelab Exercise

The students worked in groups of three, and three 6 h lab periods were required for the experiment. According to the PLTL, we suppled group leaders with reference [5] and allow them one lab period (6 h) to discuss the method with the group. Then, they chose the drugs from Ph. Eur. that could be used for the determination according to protonation and expected hydrophilicity. In the third step, they needed to cross-reference their drug list with our drug inventory (supplied by the supervisor) and decide on the drug they wanted to use for the determination. At this point (1 h to the end of the first lab period), they discussed the method and the drug of their selection with the instructor. For the drug selected, they researched the application and probable absorption body compartment, as well as all the details concerning the expected p*Ka* (for example, ionization levels at different pH of the body compartments). All the important chemical structures involved had to be drawn and discussed. All the drugs student findings by cross-referencing are given in the Figure 1. Nevertheless, it is possible to extend this on more complex compounds with more experienced students.

4.3. Procedure

In the second lab period (6 h), the students performed the actual experimental measurements. In general, the solutions of the drugs were measured by ^{1}H NMR at different pH values of the solvent, and a classical titration curve was obtained by the chemical shift vs. the pH value. All pyridine-containing drugs in our experiments had at least one aromatic proton that could be easily taken as a reference signal. The students are free to take the proton of their choice or, in advanced groups, to compare the results while using different protons. The p*Ka* value can simply be determined by graphical evaluation or by calculation. The desired value could be determined from the titration curves (e.g., Tubbs' concentric arcs method of determination of equivalence point) [16,21]. We decided

to apply this graphical determination, as up to this point in the students' education, this was the most common one. Certainly, there are other methods that can be applied, e.g., non-linear regression method, derivative method, etc. [9,16,21]. Another possibility is to calculate the mean values of the p*Ka* value determination where the chemical shifts are rapidly changing. There is no internal standard used, as the measurements are referenced to the H^2HO chemical shift, even though this is temperature-dependent at 20–25 °C, as can be found at 4.78 ppm [22]. All of the prepared solutions are adjusted using HCl and KOH solutions, as briefly described in the literature and Supporting Information [9].

The third lab period was left for the group to do literature recherché (www.reaxys.com and www.scifinder.org) and use web-based calculator to obtain the predicted p*Ka* values (https://epoch.uky.edu/ace/public/pKa.jsp). For the literature search, any web-based literature search programs could be used. Our students used sophisticated, campus-licensed programs from Heinrich Heine University, as mentioned above. They wrote a protocol and discussed their results, the literature value, and the value they obtained from the molecular modeling webpage.

5. Conclusions

The NMR-based experimental determination of p*Ka* values of different drugs in comparison to in-silico calculation with increments resulted in an increased interest and a critical scientific view of the students for data material and for physicochemical properties. The students in medicinal chemistry have achieved a deeper understanding on the importance of protonation status as well as the use of NMR as a general and versatile tool in structural determination.

Supplementary Materials: Supporting information is available online at www.mdpi.com/2312-7481/3/3/29/s1. Figure S1: 1H NMR data of Nicotinamide (extraction), Figure S2: Titration curve of Nicotinamide, Figure S3: 1H NMR data of Pyridoxine hydrochloride, Figure S4: 1H NMR data of Pyridoxine hydrochloride (extraction), Figure S5: Titration curve of Pyridoxine hydrochloride, Figure S6: 1H NMR data of Isoniazid (extraction), Figure S7: Titration curve of Isoniazid, Table S1: Chemical shifts and p*Ka* values from Nicotinamid determination, Table S2: Chemical shifts and p*Ka* values from Pyridoxine hydrochloried determination, Table S3: Chemical shifts and p*Ka* values from Isoniazid determination.

Acknowledgments: Funding for this project was kindly provided by a fund for teaching support (Lehrförderfonds) from Heinrich Heine University in Düsseldorf number L020401/15. We acknowledge the support of the Serbian Ministry of Science and Technological Development, project number III43014.

Author Contributions: A.Z. and H.S. conceived and designed the experiments; A.Z., J.J.B., A.J.S. and C.C. performed the experiments and analyzed the data; M.R., N.Z. and V.N. contributed important discussion points and technical notes; A.Z. and H.S. wrote the paper; All authors have revised the paper and agreed to publication.

Conflicts of Interest: The authors declare no conflict of interest.

References

1. Zivkovic, A.; Bandolik, J.J.; Skerhut, A.J.; Coesfeld, C.; Prascevic, M.; Zivkovic, L.; Stark, H. Quantitative analysis of multicomponent mixtures of over-the-counter pain killer drugs by low-field NMR spectroscopy. *J. Chem. Educ.* **2017**, *94*, 121–125. [CrossRef]
2. Zivkovic, A.; Bandolik, J.J.; Skerhut, A.J.; Coesfeld, C.; Zivkovic, N.; Raos, M.; Stark, H. Introducing students to NMR methods using low-field 1H NMR spectroscopy to determine the structure and the identity of natural amino acids. *J. Chem. Educ.* **2017**, *94*, 115–120. [CrossRef]
3. Bonjour, J.L.; Pitzer, J.M.; Frost, J.A. Introducing high school students to NMR spectroscopy through percent composition determination using low-field spectrometers. *J. Chem. Educ.* **2015**, *92*, 529–533. [CrossRef]
4. Danieli, E.; Perlo, J.; Blümich, B.; Casanova, F. Small magnets for portable NMR spectrometers. *Angew. Chem. Int. Ed.* **2010**, *49*, 4133–4135. [CrossRef] [PubMed]
5. Periyannan, G.R.; Lawrence, B.A.; Egan, A.E. 1H NMR Spectroscopy-Based Configurational Analysis of Mono- and Disaccharides and Detection of β-Glucosidase Activity: An Undergraduate Biochemistry Laboratory. *J. Chem. Educ.* **2015**, *92*, 1244–1249. [CrossRef]

6. Morton, J.G.; Joe, C.L.; Stolla, M.C.; Koshland, S.R.; Londergan, C.H.; Schofield, M.H. NMR Determination of Hydrogen Bond Thermodynamics in a Simple Diamide: A Physical Chemistry Experiment. *J. Chem. Educ.* **2015**, *92*, 1086–1090. [CrossRef]
7. Mobley, T.A. NMR kinetics of the SN2 reaction between BuBr and I: An introductory organic chemistry laboratory exercise. *J. Chem. Educ.* **2015**, *92*, 534–537. [CrossRef]
8. Simpson, A.J.; Mitchell, P.J.; Masoom, H.; Liaghati Mobarhan, Y.; Adamo, A.; Dicks, A.P. An oil spill in a tube: An accessible approach for teaching environmental nmr spectroscopy. *J. Chem. Educ.* **2015**, *92*, 693–697. [CrossRef]
9. Gift, A.D.; Stewart, S.M.; Bokashanga, P.K. Experimental Determination of p*Ka* Values by Use of NMR Chemical Shifts, Revisited. *J. Chem. Educ.* **2012**, *89*, 1458–1460. [CrossRef]
10. Manallack, D.T. The p*Ka* Distribution of Drugs: Application to Drug Discovery. *Perspect. Med. Chem.* **2007**, *1*, 25–38. [CrossRef]
11. Manallack, D.T.; Prankerd, R.J.; Yuriev, E.; Oprea, T.I.; Chalmers, D.K.; David, K. The significance of acid/base properties in drug discovery. *Chem. Soc. Rev.* **2014**, *42*, 485–496. [CrossRef] [PubMed]
12. Chillistone, S.; Hardman, J.G. Factors affecting drug absorption and distribution. *Anaesth. Intensive Care Med.* **2014**, *15*, 309–313. [CrossRef]
13. Repice, M.D.; Sawyer, R.K.; Hogrebe, M.C.; Brown, P.J.; Luesse, S.B.; Gealy, D.J.; Frey, R.F. Talking Through the Problems: A Study of Discourse in Peer-Led Small Groups. *Chem. Educ. Res. Pract.* **2016**, *17*, 555–568. [CrossRef]
14. Handloser, C.S.; Chakrabarty, M.R.; Mosher, M.W. Experimental Determination of p*Ka* Values by Use of NMR Chemical Shifts. *J. Chem. Ed.* **1973**, *50*, 510–511. [CrossRef]
15. Krężel, A.; Bal, W. A formula for correlating p*Ka* values determined in D_2O and H_2O. *J. Inorg. Biochem.* **2004**, *98*, 161–166. [CrossRef] [PubMed]
16. Ebel, S.; Glaser, E.; Kantelberg, R.; Reyer, B. Auswertung digitaler Titrationskurven nach dem Tubbs-Verfahren. *Fresenius' Z. Anal. Chem.* **1982**, *312*, 604–607. [CrossRef]
17. Perrin, D.D. Dissociation contants of inorganic acids and bases in aqueous solution. *Pure Appl. Chem.* **1969**, *20*, 133–236. [CrossRef]
18. Becker, C.; Dressman, J.B.; Amidon, G.L.; Junginger, H.E.; Kopp, S.; Midha, K.K.; Shah, V.; Stavchansky, S.; Barends, D.M. Biowaiver Monographs for Immediate Release Solid Oral Dosage Forms: Isoniazid. *J. Pharm. Sci.* **2007**, *96*, 522–531. [CrossRef] [PubMed]
19. Santos, T.D.A.D.D.; Da Costa, D.O.; Da Rocha Pita, S.S.; Semaan, F.S. Potentiometric and conductimetric studies of chemical equilibria for pyridoxine hydrochloride in aqueous solutions: Simple experimental determination of p*Ka* values and analytical applications to pharmaceutical analysis. *Eclet. Quim.* **2010**, *35*, 81–86. [CrossRef]
20. Friebolin, H. *Ein- und Zweidimensionale NMR-Spektroskopie*, 5th ed.; Wiley-VCH: Weinheim, Germany, 2013; ISBN 978-3-527-33492-6.
21. Tubbs, C.F. Determination of Potentiometric Titration Inflection Point by Concentric Arcs Method. *Anal. Chem.* **1954**, *26*, 1670. [CrossRef]
22. Fulmer, G.R.; Miller, A.J.M.; Sherden, N.H.; Gottlieb, H.E.; Nudelman, A.; Stoltz, B.M.; Bercaw, J.E.; Goldberg, K.I. NMR chemical shifts of trace impurities: Common laboratory solvents, organics, and gases in deuterated solvents relevant to the organometallic chemist. *Organometallics* **2010**, *29*, 2176–2179. [CrossRef]