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High Throughput pK_a Measurements

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ntroduction

)rugs are most commonly given to the body by the oral route of dministration, which leads to greater patient compliance ecause of the ease of administration. Using modern echniques of drug discovery, chemists can synthesise large umbers of molecules in a short time, but they often create olecules that are poorly absorbed. It is important that olecules with a low potential for gastro-intestinal absorption re identified as soon as possible, and discontinued from evelopment.

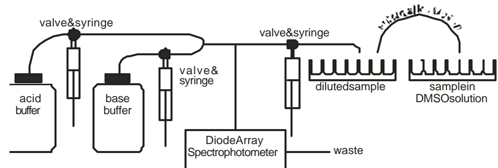
n vivo measurements of absorption are difficult and slow, and ot suitable for screening for absorption potential during drug iscovery. A number of physicochemical properties ipophilicity, solubility, size, hydrogen-bonding characteristics) ıfluence the ability of a molecule to permeate through a ıembrane by passive diffusion. Moreover, permeability, olubility and lipophilicity are pH-dependent for ionisable drugs, /hich represent about 70% of the molecules listed in the World)rug Index. Therefore, knowledge of the ionisation constant, K_a, is important. This paper details a method for the rapid ıeasurement of pK_a values.

/laterials and Methods

'he Profiler SGA (Figure 1) has been developed to address the eed for 'high throughput' measurements of ionisation (pK_a) onstants. Large numbers of pK_a measurements can be made ery quickly, using small amounts of samples. A patented rocedure was devised by creating a linear pH gradient using ıixed buffers, injecting the sample, and measuring UV bsorbance.

'igure 1. Profiler SGA (Spectrum Gradient Analyser)

igure 2 shows a schematic of the instrument. Samples are resented as 10mM solutions prepared in DMSO in 96-well lates. A flowing pH gradient is produced by mixing two buffer olutions containing mixtures of weak acids and bases that do ot absorb significantly in the UV above 250nm. 20µL of each ample is diluted with water and then injected directly into the owing gradient, which then passes through a diode array pectrophotometer measuring in the UV wavelength range. he buffer has been formulated so that its acid-base titration urve is linear over a wide pH range, such that the pH of the radient is a linear function of time. The solution pH in the ıeasurement flow cell is therefore proportional to the time lapsed since the start of gradient generation. The sample's K_a values are calculated from the change in UV absorbance at ıultiple wavelengths as a function of pH.



'igure 2. Profiler SGA schematic

nitially, acidic buffer (pH 2) is passed through the flow-cell of the liode array spectrophotometer (1 mL/min) and diluted sample s introduced at a constant flow rate (0.2 mL/min). The pH radient is then changed from pH 2 to pH 12 in a period of 2 ıinutes and is controlled by two computer controlled syringe lispensers that are inversely varied in speed whilst maintaining ı constant total flow-rate (1 mL/min). Finally, basic buffer pH 12) and sample are passed through the flow-cell for a short ime before the flow-cell is washed ready for the next sample. Alternatively, the pH gradient is run in the opposite direction rom pH 12 to pH 2.) UV spectra are recorded every second luring this time. The whole procedure of sample dilution, lowing pH gradient and flushing takes about 5 minutes.

The pH gradient was calibrated by injecting standards (at concentrations of 5×10^{-5} - 5×10^{-6} M) with known pK_a values and strong UV absorbance. Two solutions were prepared, the first containing two standards mixed together, the second containing another three standards. Each standard solution was injected into the gradient once before a (96 well) plate of samples and again at the end of the sample plate. The UV absorbance of each standard mixture was measured at 30 wavelengths, and the absorbance values were differentiated with respect to time. The positions of the peak maxima on the time axis indicate the times when the greatest change in UV absorbance at all wavelengths is occurring, which corresponds to the pK_a values of the standards. Hence, a linear calibration graph may be plotted of pK_a versus time of maximum peak height (Figure 3a and 3b).

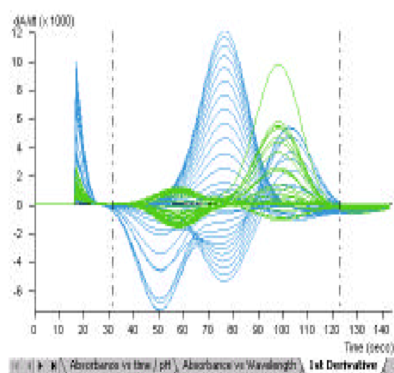


Figure 3a Derivative Spectra of Profiler SGA Standards. (Peaks at 50, 58, 77, 99 and 104 secs)

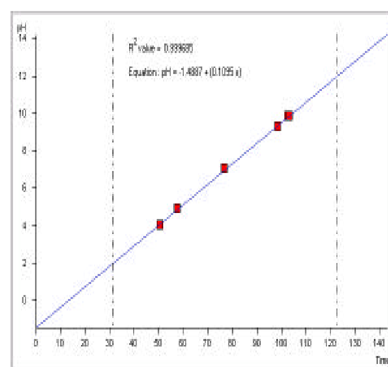


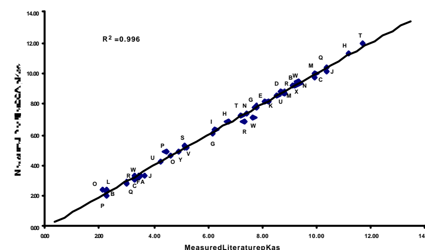
Figure 3b Calibration plot of Profiler SGA

Peaks for unknown samples can be read directly from this calibration to determine their pK_a values, where samples contain single or non-overlapping pK_a values.

In the cases of samples having multiple overlapping pK_as it is necessary to use a calculation technique known as Target Factor Analysis (TFA). To summarize, spectral data consists of absorbance values measured at different wavelengths and pH values. This can be expressed as a matrix **A**. Invoking Beer's law (**A = CE**), **A** is related to species concentration and absorptivity as follows: the **C** matrix represents the concentration of each independent light-absorbing species of the sample as a function of pH and species and the **E** matrix represents the molar absorptivity of each species (with the inclusion of the optical path length) as a function of pH and wavelength. The **A** matrix is based on measured values, but at the start of the calculation, the concentrations and absorptivities in the **C** and **E** matrixes are based on guessed values. During the calculation, the **A** matrix is deconvoluted using TFA, in which values of concentration and absorptivity are proposed interatively until **A - CE** tends to a minimum. When the minimum occurs, the pK_a values required to calculate the distribution of species shown in the **C** matrix are assumed to be the correct pK_a values.

Results and Discussion

Figure 4 shows Profiler SGA values for 25 compounds plotted against the corresponding literature values measured by pH-metric or traditional UV methods. A linear regression line has been fitted to the data with an R^2 value of 0.996, demonstrating the validity of the new technique. Moreover, the plot contains 41 pK_a values indicating that many of the compounds have two or more ionizable groups.



A=acetylsalicylic acid, B=acyclovir, C=albendazole, D=amiloride, E=clonidine, F=diazepam, G=enrofloxacin, H=famotidine, I=flumequine, J=furosemide, K=hexobarbital, L=homidium, M=hydrochlorothiazide, N=labetalol, O=nicotinincacid, P=niflumicacid, Q=nitrazepam, R=oxitetracycline, S=phenazopyridine, T=phenobarbital, U=quinine, V=sulfacetamide, W=tetracycline, X=uracil, Y=warfarin.

Figure 4. Validation of Profiler SGA

Warfarin is an example of a compound with a single ionizable group and it's pK_a value can be clearly read from the 1st derivative plot of absorbance versus pH (figure 5).

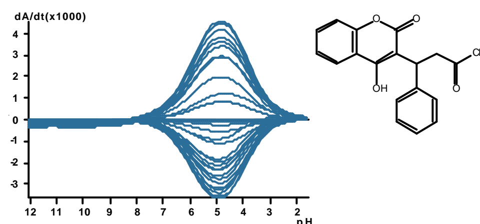


Figure 5. 1st derivative plot of absorbance vs pH for Warfarin

Labetalol, on the other hand, is an example of a compound with two highly overlapping pKa values. The phenol group causes large change in absorbance and obscures the smalle absorbance change attributed to the amine group (figure 6a and 6b). In this case, TFA is used to determine both pKa values (figure 6c and 6d).

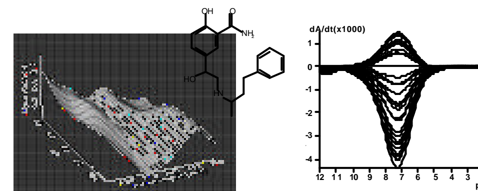


Figure 6a. & 6b. Absorbance and 1st derivative plot for labetalol

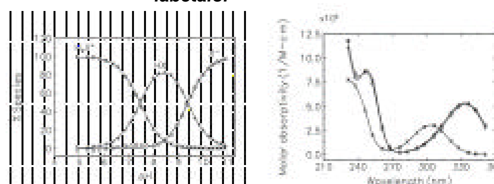


Figure 6c. & 6d. Distribution of species showing pK_a values at pH 7.4 and 9.4 and molar absorbtivity profile. Graph Symbols: H_2X^+ (x), HX(+), X^- (0).

Conclusions

The Profiler **SGA** offers the ability to deploy a "high throughput approach to the measurement of pK_a values. The instrumen works with small quantities of sample taken from 96 well plates Analysis times are about 5 minutes per sample thereb providing a throughput of several hundred compounds a day.

References

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Compounds G,T: **Sirius, unpublished pH-metric results.**

Compound L: **Sirius, unpublished pH-UV result.**

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