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Research Article

Development of a Versatile Laboratory Experiment to Teach the Metabolic Transformation of Hydrolysis

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ABSTRACT

In this paper we describe an easy, reliable, versatile and inexpensive laboratory experiment to teach the metabolic transformation of hydrolysis to Pharmacy students. The experiment does not require the sacrifice of any experimental animal, or any work with organs or tissues, and so can be implemented in a typical university chemistry laboratory. We used acetylsalicylic acid (ASA), hexyl salicylate (HS) and two enzymes, a lipase and an esterase. Since both ASS and HS liberate salicylic acid (SA) upon hydrolysis, students can evaluate the different enzymatic transformations by monitoring the amount of SA liberated. The learning outcomes are an enhanced student understanding of: (1) the process of hydrolysis; (2) the application of enzymatic transformations of molecules from food to xenobiotics; (3) the differences between the general specificity of substrate of both enzymes; (4) the concepts of the lipophilic pocket; (5) the catalytic triad and its regioselectivity in relation to the ester bond. A questionnaire was administered to participating students at three points in time: at the beginning of the module, after enzymatic hydrolysis was taught in class, and after the laboratory experiment. From an analysis of the questionnaire data we conclude that this practical helped Pharmacy students to understand these concepts.

INTRODUCTION

An in-depth understanding of drug metabolism is important for all graduates in the pharmaceutical and biomedical sectors (Silverman 2004), including medicinal chemists, pharmaceutical scientists and pharmacists. For pharmacists this is equally true whether they find employment in a hospital or community pharmacy. Not only can drug metabolism affect clinical decisions regarding pharmacotherapy, it can also elucidate the occurrence of toxic reactions in clinical trials. Numerous examples of drug interactions can also be explained on the basis of drug metabolism. In all of these situations a knowledge of drug metabolism plays a part in the day-to-day work of a pharmacist. In the future, awareness of patient metabolism will be a cornerstone of personalised medicine, in which individualised drug regimens will take into account the pharmacogenetics of each patient (Silverman 2004). There is clearly a requirement for an undergraduate experiment giving hands-on

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experience related to drug metabolism. In order to understand metabolism, enzymes must be introduced via an experiment that is straightforward, reliable, reproducible and inexpensive. Ideally, a laboratory experiment should not involve the sacrifice of experimental animals - which carries ethical connotations (Badyal & Desai 2014; Learning to Give n.d.; Dr Hadwen Trust n.d.). Neither should it require the handling of organs or tissues (Ward & Reilly 1981; Herrier *et al* 1997), which require specialised laboratory equipment.

In this paper we describe an easy and reliable experiment to teach students ester hydrolysis (Figure 1), a common metabolic phase I transformation, using acetylsalicylic acid (ASA, the original register trademark name is Aspirin®), the fragrance molecule, hexyl salicylate (HS) and two commercial enzymes (a lipase and an esterase). Techniques learnt from the experiment include UV spectroscopy, centrifugation, the preparation of a calibration curve and the use of enzymes in organic synthesis.

Fig. 1. *General representation of ester hydrolysis.*

Both ASA and HS liberate salicylic acid (SA) upon hydrolysis (Figure 2). When treated with FeCl3 (Barry & Borer 2000; Lewis 2003; Clay & McLeod 2012) the salicylic acid forms a purple complex which can be quantified by UV-visible spectrophotometry. Thus, students can evaluate the different enzymatic transformations by monitoring the SA liberated by extrapolating from a previously prepared calibration curve of SA treated with $FeCl_3$.

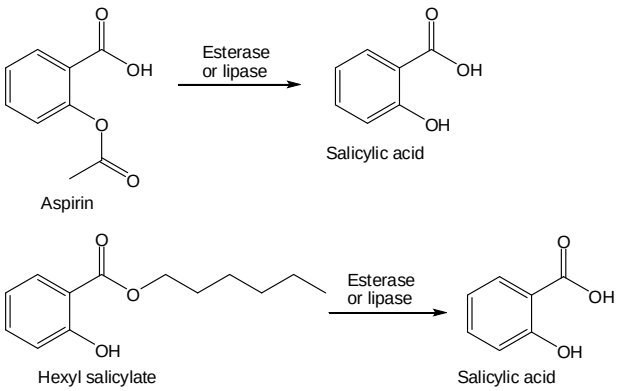

Fig. 2. *Formation of salicylic acid from ASA and hexyl salicylate via hydrolysis catalysed by lipase and esterase.*

The first learning outcome of the practical is to situate the chemical process of hydrolysis that students have seen before, into the concept of hydrolysis as a metabolic transformation of drugs and other xenobiotics.

During their 2nd year undergraduate module, 'Drug Synthesis, Metabolism and Analysis' (DSMA) our Pharmacy degree (MPharm) students have learnt that many enzymes involved in metabolic transformations of drugs are poorly selective. In this respect, cytochrome P450 3A4 (CYP3A4) is responsible for a large number of the oxidative metabolic transformations of drugs that involve CYP450 (Ogu & Maxa 2000; Silverman 2004). Furthermore, many different human esterases can cleave esters present in drugs and prodrugs even when their structures are not similar (Fukami & Yokoi 2012). Esterases can also be involved in the hydrolysis of the metabolites of xenobiotics (Fukami & Yokoi *ibid*.). These concepts are not only important for the preparation of prodrugs using esters and amides (Rautio *et al* 2008) but also interactions between food and drugs, such as the well-known interactions between grapefruit juice and many drugs (Bailey *et al* 1998; Bailey *et al* 2013; Ogu & Maxa 2000). The reinforcement of these important concepts is the second learning outcome of this experiment.

The third learning outcome is to revise and to teach students biochemical concepts such as the differences between the general specificity of the substrate of both enzymes (Fojan *et al* 2000), the presence of a lipophilic/hydrophobic pocket in esterases (such as the pig liver esterase that we used, Toone *et al* 1990) and in lipases (as described in different types of lipases, Qayed *et al* 2015; Braiuca *et al* 2009; Rengachari *et al* 2013), the fact that both active centres are not equal despite catalysing similar chemical reactions (i.e. hydrolysis of esters), the concept of the catalytic triad (Mackness & Clerc 1993) and finally how regioselectivity in relation to the ester bond (Mackness & Clerc 1993; Fuentes *et al* 2004) affects the overall performance of the enzyme reactions.

A further learning outcome, which students only discover at the end of the experiment, is an appreciation that the outcome of a reaction may be dependent on strict mechanistic requirements – and

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first impressions may be misleading if we do not understand the mechanism. This is the main reason we decided to use HS instead of a product of a similar structure, such as 2-(heptanoyloxy) benzoic acid, but with the right structure to a priori be hydrolysed faster than ASA by the lipase. A new experiment could be prepared in the future using HS and 2-(heptanoyloxy) benzoic acid.

The explanation for the last two learning outcomes is the following:

- In lectures students have learnt that esterases hydrolyse ASA. However, lipases can also hydrolyse this drug (albeit at a slower rate) despite the fact that ASA does not resemble their natural substrate. This is proof that enzymes which metabolise food can also metabolise drugs.
- Students will see that HS is metabolised by esterase, a hands-on proof that esterases metabolise a large number of substrates, which shows that the enzymes involved in drug metabolism may not be that selective after all.
- From previous biology and chemistry knowledge, students can expect that a molecule that resembles their natural substrate could be metabolised faster by the enzyme. Knowing that lipases hydrolyse long chain fatty acids attached to glycerol (triglycerides) (Mackness & Clerc 1993), and knowing that HS resembles a triglyceride because of the long hydrocarbon chain (Figure 3), many students could jump to the conclusion that it should be metabolised faster than, in this case, ASA. However, after performing the experiments, their data values will show that this is not the case. After a careful look at the structures of HS and triglycerides (Figure 3), students can see that HS does not share triglyceride features in relation to the ester that it is going to be hydrolysed. In a triglyceride the carbonyl of the ester is linked to the long chain hydrocarbon part (that fits in the lipophilic pocket), but in HS, it is orientated the other way around; this will affect the process of cleavage. Thus, it teaches students the importance of regioselectivity in chemistry and shows how fast first impressions can be deceitful.

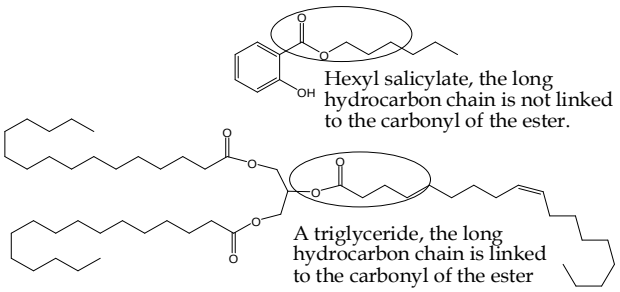

Fig. 3. *Comparative structures of HS and a common triglyceride in relation to the orientation of the ester group.*

In order to determine whether this is a versatile experiment, which could be incorporated into other undergraduate experimental practices, not only for pharmacy students but for biochemistry and pharmaceutical chemistry degrees, with pertinent changes, we decided to use a quantitative research method by means of a questionnaire designed to test student knowledge of the topics covered in the practical pre- and post-implementation. Adopting a before and after study design, the questionnaire was administered to students taking part in the practical at three points in time: at the beginning of the module ('Before Lecture'), after enzymatic hydrolysis was taught in class ('After Lecture'), and after the laboratory experiment was conducted ('After Practical'). Participants were second year Pharmacy degree (MPharm) students at the University of Huddersfield (UK). The questionnaire contained 18 items with three possible responses ('True', 'False', 'I do not know') and, to minimise response set bias, with a randomly assigned mix of correct and incorrect statements. Completion of the questionnaire was voluntary. In total, of the 80 students taking part in the practical, the questionnaire was completed by 65 students 'Before Lecture', 61 students 'After Lecture' and 59 students 'After Practical'.

The questionnaire was administered to students at three points in time. The questionnaire was administered at the beginning of the drug metabolism module in the 'Drug Synthesis, Metabolism and Analysis' module taught in the second year of their undergraduate studies, so students should at this stage only have previous knowledge from A-levels and the subjects taught in the first year to draw upon. Although some of the material taught prior to the DSMA module bears some resemblance, it does not explain in detail the

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process of hydrolysis as a metabolic first I transformation of drugs (Silverman 2004). The same questionnaire was administered to students immediately following a lecture covering the metabolic process of hydrolysis, with an expectation that students who had understood the subject from the lecture should get a better score. The questionnaire was then administered a third time at the end of the practical, to compare the impact that the practical had on reinforcing the knowledge gained from this particular lecture.

From the analysis of the data from the three questionnaires, we can see that the concepts, and overall knowledge, became increasingly clear to the participating students. Although a few (perhaps disengaged) students did not understand the concepts tested, even at the end of the practical, the overall score, and so level of understanding, was reasonably high. More information about this will be given in the "results" section.

MATERIALS AND METHODS

Materials

Esterase from porcine liver, Lipase from porcine pancreas, hexyl salicylate and tris base were purchased from Sigma-Aldrich. 15 mL falcon tubes for centrifuge were purchased from Fisher Scientific.

Although salicylic acid, acetylsalicylic acid (ASA), $FeCl_3$ and MeOH were currently in stock in our institution, they can be purchased from Sigma-Aldrich.

Pictures used in the practical illustrations were created using Discovery Studio 4.5 Visualiser, together with pictures from the protein data bank.

To stir the samples and perform the enzyme reactions we used a shaking water bath Stuart SBS40 with 15 mL tube racks as an incubator.

The centrifugation was performed in a Mistral 3000i centrifuge.

Experimental procedure

The original experimental procedure handed out to the students contained a short explanation of the objectives of the practical, a short introduction to enzymatic hydrolysis, the concept of lipases and esterases, the concept of the active site of an enzyme, the concept of a lipophilic pocket and a short introduction to the experiment.

The reactions involved are described in Figure 2. The analysis of enzyme activity was performed by analysing the amount of salicylic acid liberated in each reaction by means of the phenol test that produces a deep purple coloured solution (Figure 4) easily checked by UV-visible spectrophotometry (Barry & Borer 2000; Lewis 2003; Clay & McLeod 2012).

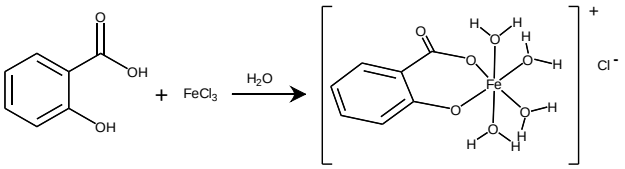

Fig. 4. *Formation of the purple salicylic acid-$FeCl_3$ complex.*

The solids should be weighed in the balance room. However, hexyl salicylate is a liquid that must be measured in the fume cupboard using the micropipette provided. Gloves and goggles should always be worn. The tips of the micropipette needed to be disposed of in the provided container. Please see the "Hazards" section for more details.

The experimental procedure was as follows:

The practical was delivered to two classes with 40 students in each. The students (n=80), in teams of two, were required to prepare four reaction mixtures in a falcon tube (15 mL), label them properly, and put them in the incubator at 60 rpm at 37 °C for 60 min. The four reaction mixtures were as described below:

- Reaction mixture 1: 30 mg of esterase and 30 mg of ASA placed in 10 mL of Tris buffer 0.1 M, pH 8.0 at 37 °C for 60 min.
- Reaction mixture 2: 30 mg of lipase and 30 mg of ASA placed in 10 mL of Tris buffer 0.1 M, pH 8.0 at 37 °C for 60 min.
- Reaction mixture 3: 30 mg of esterase and 38.5 µL (37 mg) of hexyl salicylate placed in 10 mL of Tris buffer 0.1 M, pH 8.0 at 37 °C for 60 min.
- Reaction mixture 4: 30 mg of lipase and 38.5 µL (37 mg) of hexyl salicylate placed in 10 mL of Tris buffer 0.1 M, pH 8.0 at 37 °C for 60 min.

While the samples were stirring each team had to build a seven point calibration curve of salicylic acid at the start of each practical (described below). For this they first prepared a standard solution of

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salicylic acid 50 mg. L-1 (0.36 mM) in MeOH/H_2O mixture 1:1 (v/v) in a 100 mL volumetric flask and then followed the steps described below. They were provided with 0.03 M $FeCl_3$ solution and also provided with 0.1 M Tris buffer solution adjusted at pH = 8. The steps to be followed were:

1. Prepare 100 mL of MeOH/H_2O mixture 1:1 (v/v).
2. Dissolve 5 mg of salicylic acid in 100 mL of a mixture of methanol/H_2O 1:1 (v/v) using one 100 mL volumetric flask. Label it properly.
3. Prepare 7 different solutions of increasing concentration of standard salicylic acid solution (SA), placing each in one properly labelled 10 mL volumetric flask. For this procedure you will add the following to each flask:
   - 3.1. Flask 1 = 0 mL of standard salicylic acid solution + 0.5 mL of $FeCl_3$ 0.03 M solution + 1 mL 0.1 M Tris buffer solution
   - 3.2. Flask 2 = 1 mL of standard salicylic acid solution + 0.5 mL of $FeCl_3$ 0.03 M solution + 1 mL 0.1 M Tris buffer solution
   - 3.3. Flask 3 = 2 mL of standard salicylic acid solution + 0.5 mL of $FeCl_3$ 0.03 M solution + 1 mL 0.1 M Tris buffer solution
   - 3.4. Flask 4 = 3 mL of standard salicylic acid solution + 0.5 mL of $FeCl_3$ 0.03 M solution + 1 mL 0.1 M Tris buffer solution
   - 3.5. Flask 5 = 4 mL of standard salicylic acid solution + 0.5 mL of $FeCl_3$ 0.03 M solution + 1 mL 0.1 M Tris buffer solution
   - 3.6. Flask 6 = 5 mL of standard salicylic acid solution + 0.5 mL of $FeCl_3$ 0.03 M solution + 1 mL 0.1 M Tris buffer solution
   - 3.7. Flask 6 = 6 mL of standard salicylic acid solution + 0.5 mL of $FeCl_3$ 0.03 M solution + 1 mL 0.1 M Tris buffer solution
4. Fill each flask up to 10 mL with MeOH/H_2O mixture 1:1 (v/v).
5. Measure the UV-visible absorbance of each flask's solution at 580 nm. The blank solution will be MeOH/H_2O mixture 1:1 (v/v).

After these five steps, the students had to plot a calibration curve on graph paper provided, writing samples 1 to 6 on the x-coordinate (or abscissa) in order of increasing concentration of salicylic acid and on the y-coordinate (or ordinate) plot the UV absorption similar to the one described in Figure 5.

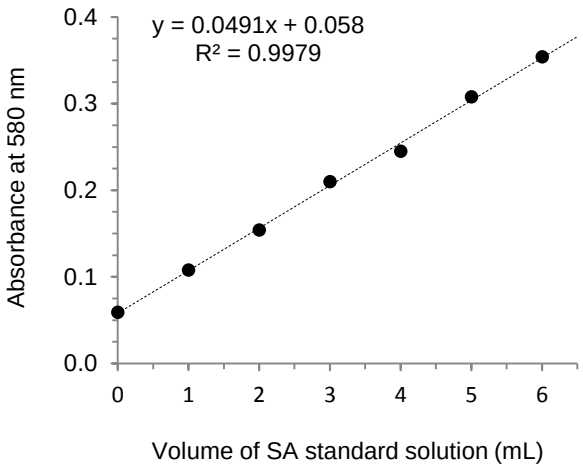

Fig. 5. *Example of calibration curve of the different concentrations of salicylic acid – $FeCl_3$ complex at 580 nm. Flasks 0-6 with absorbances of 0.059, 0.108, 0.154, 0.21, 0.245, 0.308 and 0.354.*

Once they had plotted the calibration curve, the students had to prepare 4 vials with 5 mL of MeOH each. After the reactions had been stirring for 60 min, they had to put the tubes in ice and add the 5 ml of methanol to each of them to precipitate the protein. After that, each labelled tube had to be centrifuged for 10 min at 2500 rpm, 4 ^oC using a MSE Mistral 3000i centrifuge or equivalent. Back in the chemistry laboratory, students were then instructed to get 1 mL of supernatant from each tube, put it into a 10 mL volumetric flask, add 0.5 mL of $FeCl_3$ solution, and fill the flask up to 10 mL with MeOH/H_2O 1:1 (v/v).

The student teams had then to measure the UV-visible absorbance at 580 nm with MeOH:H_2O 1:1 (v/v) as blank and then plot their values for each reaction on the calibration curve.

In the conditions used, no hexyl salicylate (which has a higher density than water) should remain in solution, so students' results will only be representative of the amount of salicylic acid presented in the sample, not the phenol in HS (this is clearly visible in the centrifuged). The amount of salicylic acid present is just directly related to the ability to hydrolyse esters of these enzymes.

After the experiments students were asked to hand in the graphic and - in our case - the completed questionnaire to the demonstrator before leaving the laboratory. They were also required to complete a coursework assignment based on the practical which entailed answering a set of questions (detailed below) online (with a deadline of one week following

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the end of the practical). The assignment was graded as part of the module coursework. For this assignment, a document with the question's model answers is included in Annexe I. As a summary, esterase hydrolyses ASA and HS in a similar yield. Also, the chemical yields of esterase reactions are higher than those of lipase reactions (with an even higher difference if we take into consideration the number of units of each enzyme). Furthermore, when we made a reaction between porcine pancreas lipase in these conditions with ASA and HS, the higher yield corresponded to AS, despite the long hydrocarbon chain of HS. The lack of yield for the reaction of HS with lipase is explained later. The higher yield for the reaction between ASA and lipase vs HS and lipase could be related to the lower solubility in water of HS, which although could be thought to be the same situation as their natural substrate of lipase, bile salts acting as an emulsifier are needed for hydrolysis of triglycerides in vivo (Bemback *et al* 1990) and in vitro (Bemback *et al* 1990; Mattson & Beck 1955) and we did not use these in our experiment.

Different types of question (*e.g.* about the type of specificity of these enzymes) could be asked, depending on the academic degree and level of students. However, for our students the online coursework assignment questions we designed were:

1- Which enzyme has shown higher activity? [0.5 marks]
2- Which molecule has been hydrolysed more extensively? [0.5 marks]
3- Are the enzymes involved in drug metabolism selective? [0.5 marks]
4- Are the enzymes that metabolise food selective? [0.5 marks]
5- Why do we use 30 mg of ASA but 37 mg of hexyl salicylate? [0.5 marks]
6- Were you expecting these results? Why? [0.5 marks]
7- An important concept in enzymology is the catalytic triad. Describe in detail the catalytic triad. [2 marks]
8- Describe how the concept of the catalytic triad explains your practical's results. [2 marks]

It is worth noting that no participating undergraduate Pharmacy degree (MPharm) students correctly answered question 8, despite having seen the concept of regioselectivity the year before. This was surprising to the authors who do not have an explanation for this. However, we believe that if the same question was asked to students from a degree with a greater chemical content it should be answered correctly by many students.

A plausible explanation of this difference in reactivity is that in HS, the enzyme has to approach the ester by the aromatic part, for all the atoms to fit the catalytic triad, not by the long chain (like in their natural substrate) as a quick view may suggest. This fact does not happen with esterase and hence the higher yield hydrolytic of hydrolysis (even taking into consideration that the lipase used has a larger number of units than the lipase). This is a clear example of the importance of paying attention to detail in science which can be used as an example for students and, as mentioned before, is a non-written learning outcome from this practical.

As a more detailed explanation, pig liver esterase's active centre has two hydrophobic pockets, one large and one small, in a particular conformation next to a serine residue (Toone *et al* 1990), and in our opinion the large one could accommodate the aromatic ring (Figure 6) on both molecules and hence the better yields.

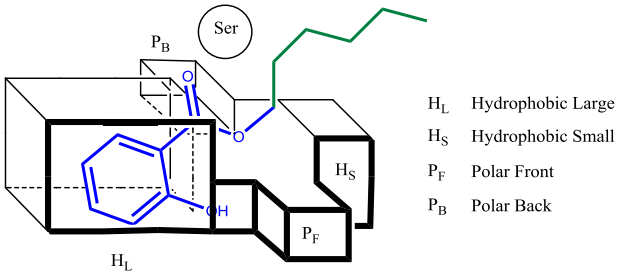

Fig. 6. *Hydrophobic pockets configuration in porcine liver estearse (adapted from Toone et al 1990).*

However, lipases (like the porcine pancreas esterase used in this experiment) display an enhanced content of non-polar residues in places that are largely accessible to the solvent (Fojan *et al* 2000), despite the fact that both hydrolyse esters and have similarities in the amino acid composition (Fojan *et al* 2000). Because of the different orientation of the ester in HS compared with triglycerides (Figure 3), prior to hydrolysis, the long chain does not enter the hydrophobic ester long chain, and has to be the aromatic ring. A not-to-scale quick representation is depicted in Figure 7, and compared with a long chain

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substrate as seen in the protein data bank. This may distort the conformation of the enzyme.

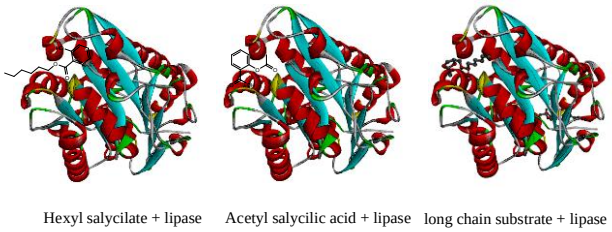

Fig. 7. *Schematic representation of the regioselective lipase (not to scale).*

If we assume that the hydrophobic pocket of porcine pancreatic lipase resembles that of bacterial monoacylglycerol lipase (bMGL) (Rengachari *et al* 2013) then the pocket will comfortably accommodate long chain hydrocarbons (Figure 8) but is too narrow to easily accommodate an aromatic ring, distorting the pocket and delaying the hydrolysis process.

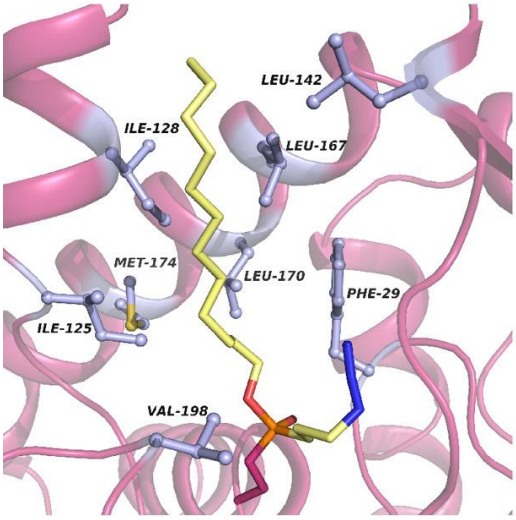

Fig. 8. *Binding of the alkyl chain of a substrate analog in the substrate binding pocket of bMGL (adapted from Rengachari et al 2013). Due to the proximity of the Ile, Leu and Met residues, an aromatic ring will not be as easy to accommodate as the lipase that we have used.*

Hazards

After performing a formal risk assessment and an extensive bibliography search of the reagents needed for the practical, we decided to minimize any risk by following the procedures described below.

HS is volatile and a mild irritant, so it must be measured out in the fume hood. Pipette tips have to be discarded in a container containing soapy water. Because HS is a liquid, for practical purposes, the measurement is done by volume using micropipettes, rather than by weight. This speeds up the process and reduces the chance of contact.

ASA is also slightly irritant but it is not volatile and the chance of contact with skin, eyes and mucoses is very slim. However, we obliged our students to follow laboratory safety procedures (coat, goggles, gloves) when handling ASA.

Lipase and esterase enzymes are innocuous, although we obliged the students to handle them with care. Esterase from porcine liver needs to be kept in the freezer at (-20 ^{o}C) and Lipase in the fridge (at 2-8 ^{o}C). So, once the students had finished measuring, the enzymes were put back in the fridge or freezer.

Classroom settings

This experiment was performed by second year Pharmacy degree (MPharm) students as part of the "Drug Synthesis, Metabolism and Analysis" course. The experiment was set up for 2 x 3.5 h sessions, each of 40 students, in a chemistry laboratory equipped with chemical fume hoods. The room contained enough laboratory bench space and fume hoods for up to 56 students.

Students worked in 20 pairs and each pair had access to 4 Falcon tubes (15 mL), 7 volumetric flasks of 10 mL, one UV-visible spectrophotometer Jenway 6350, 5 plastic UV cuvettes, 1 piece of graph paper, 1 volumetric of 100 mL, and 1 beaker (250 mL). There were also 10 SciPette micropipettes of 200 mL (2 per bench) and 10 SciPette micropipettes of 1 mL (2 per bench). There were containers with soapy water in each fume cupboard to dispose of the used tips that may contain traces of hexyl salicylate. High accuracy balances were set up in a separate room close to the laboratory and students had to go there to weigh ASA and AS.

The shakers/incubators were kept in a fume cupboard in the chemistry laboratory. Because our laboratory does not have a centrifuge, we used the centrifuge situated in the biology laboratory. A demonstrator carried the samples to the biology laboratory at intervals for centrifugation, then returned them to the chemistry laboratory for analysis of supernatants.

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RESULTS AND DISCUSSION

In general, students preferred this practical over traditional synthetic organic experiments they had undertaken in the past (ascertained from anecdotal reports by students). Some minor problems occurred (mainly from long waiting times) when the practical was first performed but in the second class everything ran smoothly.

The esterase hydrolysed ASA and HS in similar yield. Also, the chemical yields of esterase reactions were higher than for the lipase reactions (an even higher difference if we take into consideration the number of units of each enzyme). Furthermore, when we made react porcine pancreas lipase in these conditions with ASA and HS, the higher yield corresponded to ASA, despite the long hydrocarbon chain of HS.

From the analysis of questionnaire data (Table 1) we found that there were more correct responses in the test administered 'After the Practical' (mean = 13.61) than in the test 'After the Lecture' (mean = 10.56). An independent t-test showed this difference was statistically significant ($t = -5.723$, $df = 118$, $p < 0.001$). (Although the same population completed each questionnaire an independent, rather than paired, t-test was computed as cases were not matched due to, for practical reasons, identifiers not being used on the questionnaires). There were also more correct answers for many test items 'After the Lecture' compared with 'Before the Lecture', showing an impact of the lecture on test scores. However, this did not reach statistical significance. This means that the effect of the practical (with prior lecture) on test scores was greater than the effect of the lecture alone. Thus we can conclude that the practical had a positive effect on test scores. When interpreting this result it should be noted that the 'After practical' score actually denotes the test scores 'After practical AND lecture', and not the effect of the practical alone, since the effects of each intervention were not examined separately in the current case study. Nonetheless, the results indicate a positive effect of the practical on student learning.

Please see full table of questionnaire data in the annexed information.

Tips and possible problems

It is best that the centrifugation is carried out by the demonstrator, balancing the tubes properly, so the students just give the properly labelled samples to the demonstrator.

Table 1. Total test scores (mean scores)

Group	No. of students	Mean	Std. Deviation
Before Lecture	65	10.1	7.3
After Lecture	61	10.6	3.1
After Practical[a]	59	13.6	2.8

[a] 'After practical' score is 'After practical AND the lecture'

No hexyl salicylate should remain in solution, so the results from the phenol test will only be representative of the amount of salicylic acid present in the sample, as hexyl salicylate is denser than water ($d = 1.04$ g/mL at 25 °C) and remains at the bottom together with the precipitated protein. Students were reminded not to shake the samples. However, in the occasional event that a student team dropped (or shook) a sample of hexyl salicylate, the value of hydrolysis was higher than expected.

60 min was sufficient for the students to draw the calibration curve and for the enzymes to hydrolyse the esters in a noticeable manner.

Some students had problems measuring 5 mg of salicylic acid (to prepare a 100 mL solution). This is because of their inexperience in transferring small amounts, as explained by students to the main demonstrator, and this may have been part of the reason for a slight disparity in the curves. It is recommended that the solution be freshly prepared in advance by a technician (50 mg in 1000 mL) and 10 or 20 mL be given to the students.

CONCLUSIONS

We have developed an easy, reliable, versatile and inexpensive laboratory experiment to teach the metabolic phase I transformation of hydrolysis to pharmacy students that does not require the sacrifice of any experimental animal. From an analysis of the questionnaire data, we observed that the effect of the practical (with prior lecture) on test scores was greater than the effect of the lecture alone. Thus we can conclude that the practical had a positive effect

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on test scores and helped students to understand this metabolic process.

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