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Flow chemistry synthesis of zolpidem, alpidem and other $GABA_A$ agonists and their biological evaluation through the use of in-line frontal affinity chromatography†

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The flow of information between chemical and biological research can present a bottleneck in pharmaceutical research. Tools that bridge these disciplines and aid information exchange have therefore clear value. Over the last few years, both synthetic chemistry and biological screening have benefited from automation, and a seamless chemistry–biology interface is now possible. We report here on the use of flow processes to perform synthesis and biological evaluation in an integrated manner. As proof of concept, a flow synthesis of a series of imidazo[1,2-*a*]pyridines, including zolpidem and alpidem, was developed and connected to a Frontal Affinity Chromatography screening assay to investigate their interaction with Human Serum Albumin (HSA).

Introduction

The power of modern day science is more often than not advanced when different disciplines can be exploited in a synergistic fashion. Also true is that discoveries in one field through various levels of innovation can have significant impact on other disciplines. Bottlenecks and problems usually arise however when there are disconnections between the sciences. Typical of this problem occurs in drug discovery processes where wastage and extended timelines can lead to frustration and poor information flow between disciplines. Tools therefore that can help build bridges will clearly have value. Given that continuous methods and flow processing for both chemical synthesis[1] and biological evaluation[2] using various microreactors or microfluidic chips are now well established it makes sense to integrate these devices to produce coordinated systems to make and screen molecules.

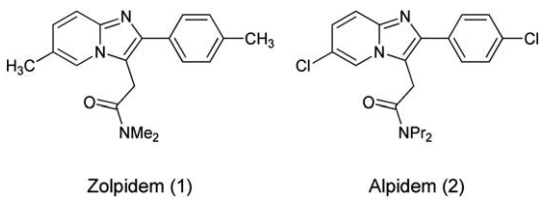
Reported here are the beginnings of this concept where we have developed a suitable flow chemistry synthesis platform and investigated its compatibility with Frontal Affinity Chromatography (FAC) as a potential in-line screening device.

We have already demonstrated the power of flow chemistry methods to deliver multi-step syntheses of drug substances and natural products.[3] However until now, we lacked a direct in-line and practical screening method that would be dynamic in character and compatible with a flow chemistry environment.

The flow preparation of imidazo[1,2-*a*] pyridines

The semi-automated platforms can optimise reaction conditions quickly and circumvent scale-up issues such as avoiding large volumes of hazardous materials or better control over kinetics of the reaction and thereby conforming to a more chemically sustainable agenda. Robotic autosamplers and fraction collectors can accelerate compound library synthesis, and in-line purification methods using immobilised systems can reduce the overall processing time significantly.

Here, we show that a series of $GABA_A$ agonists, such as zolpidem, alpidem (**1** and **2**, Fig. 1) and other analogues can automatically and quickly be synthesised using a computer-controlled flow chemistry platform. After each derivative is prepared, it is evaluated for biophysical data using a Frontal Affinity Chromatography system (see later). All compounds were obtained following the general flow chemistry route described below (Scheme 1).



Zolpidem (1) Alpidem (2)

Fig. 1 Structures of zolpidem (**1**) and alpidem (**2**).

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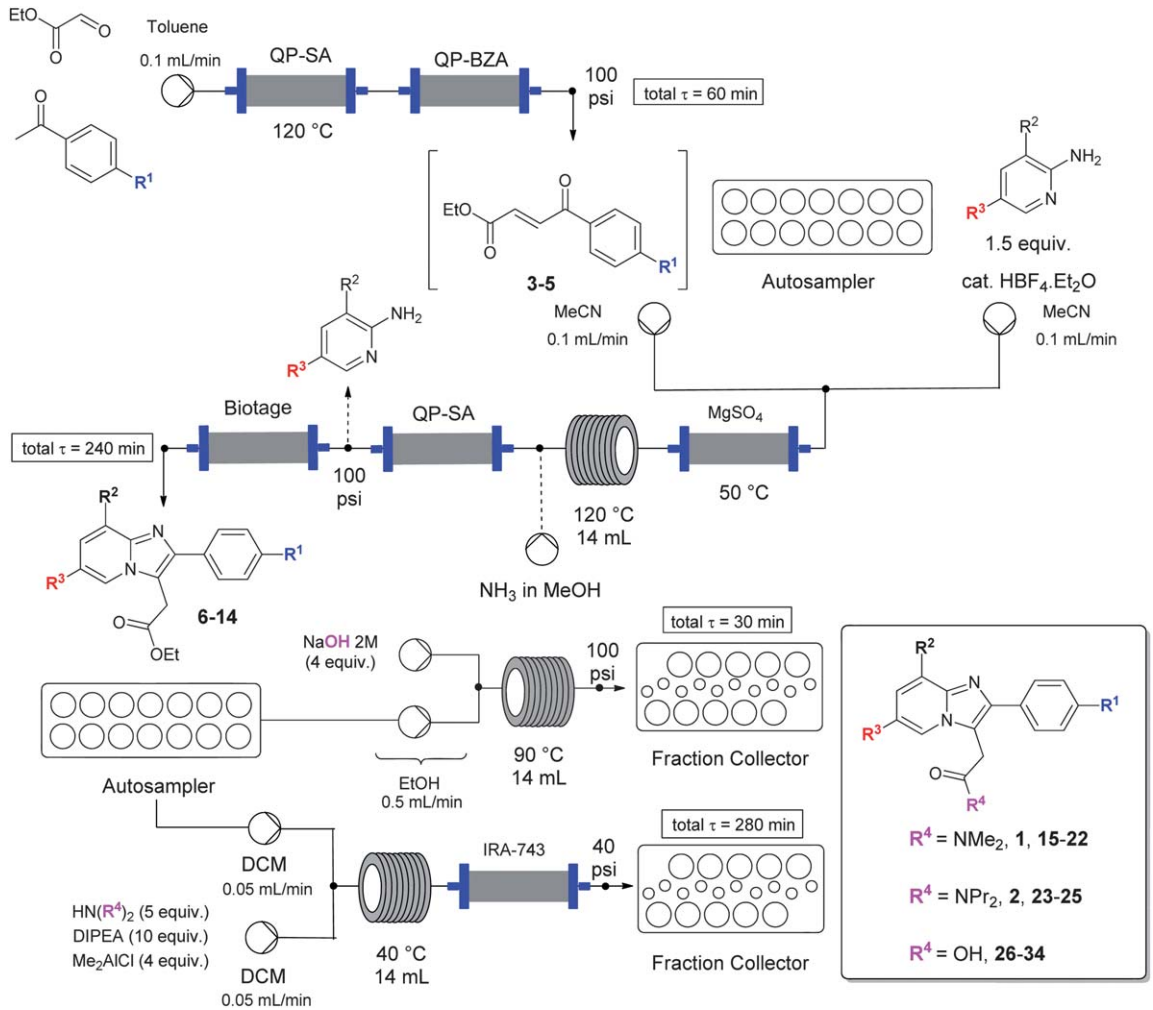


Scheme 1 Flow synthesis of imidazo[1,2-*a*]pyridines.

Zolpidem (**1**) and alpidem (**2**) are agonists of $GABA_A$ receptors,[4] and belong structurally to the class of imidazo[1,2-*a*] pyridines. These heterocyclic scaffolds are found in molecules displaying anticancer, antimicrobial and antiviral properties, and are therefore attractive targets for medicinal chemistry programmes,[5] including using flow chemistry technologies.[6]

Scheme 1 summarises our efficient flow chemistry preparation of imidazo[1,2-*a*]pyridine derivatives. In the first step the acid catalysed condensation between ethyl glyoxylate and acetophenone analogues leads to unsaturated ketone intermediates (**3–5**) which can be repetitively produced on multigram scale. The acetophenone derivatives with an excess of glyoxylate are pumped through the reactor cartridges *via* injection loops. The condensation between the substrates happens in reactors packed with polymer supported sulfonic acid resin (QP-SA)[7] at 120 °C, with typical residence time of around 25 minutes based on combined flow rate of 0.1 mL min^{-1}. The exiting stream was cleaned by passage through a column of QP-BZA,[7] a supported benzyl amine which scavenges any excess of glyoxylate. The combination of these two heterogeneous in-line reagents makes the process free from any further downstream work up and purification (repeated at 10 mmol scale every 3 hours). The products (**3–5**) are collected and transferred to the next synthesis step. Using an autosampler the unsaturated ketone (**3–5**) and a slight excess of an aminopyridine derivative are then pumped through a column packed with dehydrating agent and heated at 50 °C to promote ketimine formation, and subsequently are transferred to a 14 mL reactor at 120 °C leading to anticipated 5-exo cyclisation. There are clear advantages of using these flow systems compared to a traditional batch process. For example, the dispensing of different aminopyridines into the reactors can be achieved automatically to make a diversified collection of compounds. The use of the flow process permits safe superheating of the solvent under back-pressure control. Indeed, using conventional heating conditions, the reaction typically takes 24 hours whereas the superheated process in the flow reactor takes around 4 hours to go to completion. The same results can be achieved by using microwave reactors, however it should be noted that scaling up microwave reactions is not always easy. The reaction mixture then passes through a column packed with supported acid resin (QP-SA) which captures the excess but valuable aminopyridine. This can be recovered later by injecting ammonia in methanol to release the bound material. Although amenable to

automation, this one step method results in lower yields and presence of Michael addition by-products that requires column chromatography to obtain pure products (**6–14**).

The final step of the synthesis is where structural diversity (and more hydrophilicity) can be introduced to the imidazopyridine scaffold, using the autosampler to control two distinct processes. The first reaction involves the conversion of the esters to the corresponding amides using dimethyl aluminium chloride as an enabling reagent.[8] Similar flow transformation has already been described using microfluidic devices[9] although these authors mentioned that the reaction can be accelerated by heating whereas for the batch procedure sub-zero temperatures are often encountered. In our hands degradation was observed at high temperatures, consequently the reaction was carried out at 40 °C over 280 min. Using flow chemistry technologies therefore has enabled us to perform these reactions faster compared to typical overnight batch reaction conditions. It has also simplified the work-up of these reactions. A column packed with IRA-743 (polyol resin) and a plug of silica conveniently removes the aluminium salts and the excess of base. As long as total anhydrous conditions are maintained throughout the synthesis, a library of corresponding amides can be prepared *via* the autosampler in a semi-automated fashion (**1**, **2**, **15–25**). The final process involves a saponification procedure to afford the corresponding carboxylic acids (**26–34**). Using this platform, **22** analogues of $GABA_A$ agonists were prepared in a very short period of time (4 days). These compounds can be collected by simply removing the solvent and are manually prepared for the FAC assay. Alternatively, the Uniqsis flow system can take aliquots of 10 microlitre for each reaction output which can then be introduced directly into FAC assay upon proper dilution (*vide infra*).

Frontal affinity chromatography

Frontal Affinity Chromatography (FAC) is a biophysical method originally used to study the interaction between carbohydrates and lectins,[10] but now is applied to a broad range of biological targets thanks to advancements in molecular biology.[11,12] In FAC, the molecule of interest is continuously infused through a column containing an immobilised target biomolecule and retained, in proportion to its affinity (Fig. 2).

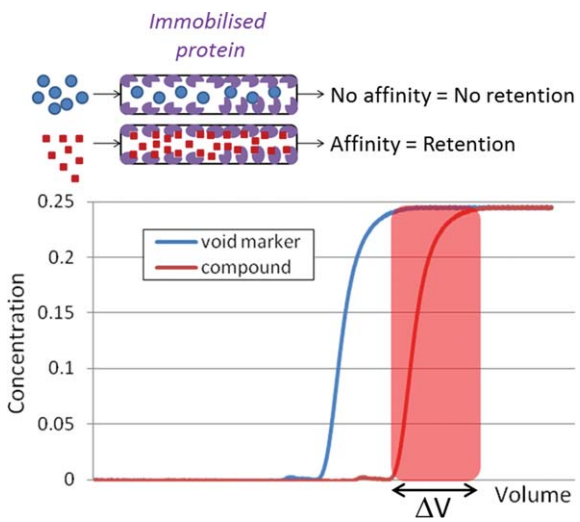


Fig. 2 Principle of FAC.

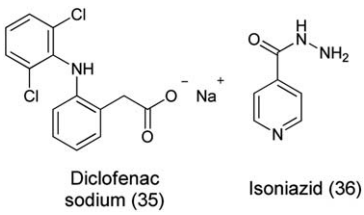


Fig. 3 Structures of diclofenac sodium (**35**) and isoniazid (**36**).

To calibrate the system, a void marker – a molecule with no affinity – is first used to define the column's reference retention characteristics.

$$[A_0]\Delta V = B_t[A_0]/([A_0] + K_d) \quad (1)$$

Binding constants (K_d) can then be calculated from eqn (1) using breakthrough volumes of the analyte (ΔV, difference of retention between the analyte and the void marker), where $[A_0]$ is the total concentration of analyte and B_t the amount of immobilised target. As the mathematical models used are well validated[13] a software programme can be readily developed to enable automation.

As proof of concept for the screening experiments, Human Serum Albumin (HSA) was chosen as the biological target. This protein is available in reasonable purity and at low cost. Being the most abundant protein in human blood plasma, it is involved in many roles, including carrying hormones and other compounds, and also maintaining cellular osmotic pressures. Most drugs bind to HSA to some extent and this level of binding determines the amount of free drug in blood which in turn affects pharmacokinetic properties of the compound. As a result, HSA-binding data is available for most drugs and many biologically relevant molecules.[14] These data help considerably with the validation of our assay system.

Here, we chose diclofenac sodium (**35**) and isoniazid (**36**) as two markers to test the system (Fig. 3). Diclofenac sodium (**35**), a NSAID drug, is known for having a very high (>99%) binding to plasma protein.[14] On the other hand, isoniazid (**36**), a medicinal agent used for tuberculosis, is reported to have no affinity for HSA.[14] Consequently in any FAC assay isoniazid (**36**) should readily pass through the column whereas diclofenac sodium (**35**) would be highly retained.

For our proof of concept study, we therefore used a commercially available chiral HSA column. However, due to the high amount of immobilised HSA on this column, even high concentrations of diclofenac sodium were retained for an exceptionally long time which made the use of this column in the present FAC assay impractical. A fraction of immobilised HSA was therefore repacked in a guard column (15 μL volume) and the column was attached to a standard Agilent 1100 HPLC system. When 600 μL of 62.5 μM solutions of diclofenac

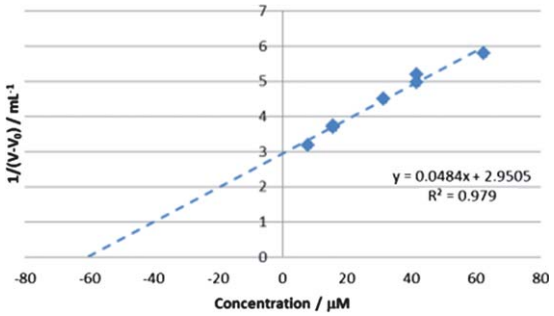


Fig. 4 Graphical determination of binding affinity (K_d) of zolpidem (**1**) for HSA and the amount of immobilised HSA (B_t) on the column.

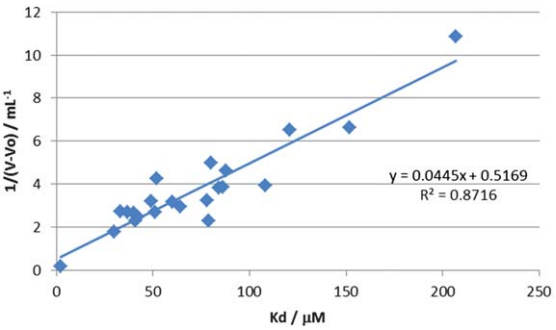


Fig. 5 Ranking method: correlation between affinity constants K_d and breakthrough volumes ($V - V_0$).

sodium (**35**) and isoniazid (**36**) in Phosphate Buffer Saline (PBS) were infused through the column at 50 µL min^{-1} we were delighted to see that isoniazid was not retained at all whereas diclofenac sodium (**35**) displayed a retention volume of 838 µL.

Initially, different concentrations of zolpidem (**1**) in PBS were prepared and infused in triplicate through the HSA column. The breakthrough volumes (ΔV) were calculated. The inverse ΔVs were plotted *versus* concentrations to give the dissociation constant (K_d) of zolpidem (**1**) for HSA and the amount of immobilised protein (B_t) (Fig. 4). K_d was found to be 61 µM and in accordance with previously published data[14,15] and B_t was 20.7 nmoles. For details, see Experimental section.

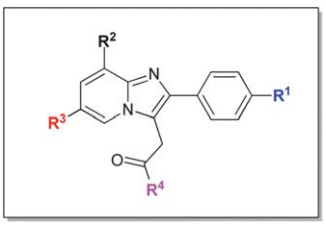
The K_d values for the synthesised $GABA_A$ analogues, were then determined one by one as they were prepared (Table 1).

As the B_t of the column is known, and assuming that all these compounds interact with the same binding site(s), only one injection of one concentration would be enough to calculate the K_d, however by injecting more than one concentration another important advantage for this assay system can be gained as well as K_d: the B_t can also be calculated independently for each analogue. This provided a built-in self-check for the assay which can be very helpful especially in early stages of discovery. If a compound has a B_t which is significantly different to others, this can be an early indication of an error. This self-check system of the assay can prevent false negative or positive results being obtained. UV absorbance of each concentration can also provide clue about the accuracy of the performed assay. If it is lower or higher than expected it can be an indication of error in sample preparation and again is a useful check for the screening protocol.

Alternatively, a single injection can be used to rank compounds according to their affinity. This is particularly useful when large numbers of compounds are being assayed and has mostly been used in a qualitative manner when screening mixtures for hit identification.[12] Furthermore, when $[A_0] \ll K_d$, then eqn (1) can be simplified and expressed as: $1/\Delta V = K_d/B_t$,[13] which makes it independent from the concentration of analyte. This is of utmost practical value when a fully automated system is developed as there is no need to know the exact concentration of the sample, as long it is diluted enough. To the best of our knowledge this is the only concentration-independent bioassay which makes it a perfect candidate for integration with flow chemistry platforms.

Here the Uniqsis FlowSyn system transfers an aliquot (10 µL) of each compound which can then be introduced into FAC assay upon proper dilution. As expected, the results of this ranking approach were in accordance with Table 1. There is indeed a satisfactory linear correlation between the affinity constants and the inverse of breakthrough volumes (Fig. 5), showing that outputs from the flow synthesis platform can be used directly to perform the binding assay.

Table 1 Affinity of $GABA_A$ ligands for HSA



Compound	R_1	R_2	R_3	R_4	K_d (µM)
15	H	H	H	NMe_2	207
16	H	H	CH_3	NMe_2	121
17	H	H	Cl	NMe_2	108
18	CH_3	H	H	NMe_2	152
1	CH_3	H	CH_3	NMe_2	60
19	CH_3	H	Cl	NMe_2	88
20	CH_3	Br	CH_3	NMe_2	84
21	Cl	H	CH_3	NMe_2	78
22	Cl	H	Cl	NMe_2	64
23	H	H	H	NPr_2	41
24	CH_3	H	H	NPr_2	79
25	CH_3	H	CH_3	NPr_2	42
2	Cl	H	Cl	NPr_2	2
26	H	H	H	OH	86
27	H	H	CH_3	OH	80
28	H	H	Cl	OH	52
29	CH_3	H	H	OH	49
30	CH_3	H	CH_3	OH	30
31	CH_3	H	Cl	OH	40
32	CH_3	Br	CH_3	OH	33
33	Cl	H	CH_3	OH	51
34	Cl	H	Cl	OH	37

Conclusions

In summary we have demonstrated that by the use of enabling flow chemistry methods we can assemble a collection of imidazo[1,2-*a*]pyridines as potential $GABA_A$ agonists including zolpidem (**1**) and alpidem (**2**), and evaluated these for binding properties to HSA using frontal affinity chromatography. This has been achieved *via* equipment in a footprint of one standard fume cupboard. We observed that affinities of zolpidem (**1**) and alpidem (**2**) are in accordance with literature values[14,15] and could obtain simple structure–activity relationship using a series of related analogues. The results of this study correlate with known drug-serum protein interactions.[16] While we have not yet fully integrated the synthesis with the biological assay, the molecular components we have employed will form the basis for future developments towards this goal. We anticipate that the concentration-independent nature of FAC assay will greatly facilitate the integration of biology and chemistry which will ultimately lead to an automated drug discovery platform.

Experimental section

Synthesis

Details for synthesis and full characterisation of all compounds can be found in ESI.†

Frontal affinity chromatography assays

General considerations. Phosphate Saline Buffer (PBS), diclofenac sodium salt, isoniazid and immobilised HSA on silica gel (Ø 5 μm, Suppelco) were bought from Sigma-Aldrich. PBS was sterilised by filtration (Millipore, 0.22 μM) prior to use. DMSO (Alfa Aesar) was used without any further purification. The 15 μL guard column (1 mm × 2 cm) was purchased from Kinesis. FAC assays were run on an Agilent 1100 HPLC system using PBS as the eluent.

Retention volumes were calculated with an in-house Excel Macro file, which determines the time between the injection point and the point when the concentration reaches 50% of the plateau.

Methods. The HPLC system was set-up to inject 600 μL of each concentration of analyte at 50 μL min^{-1} for 40 min. Three wavelengths were simultaneously monitored at 220 nm (analyte or DMSO), 254 nm (analyte) and 262 nm (analyte) using a Diode Array UV Detector.

Serial dilutions of each compound were prepared from a 100 mL stock solution at 125 μM in PBS. When solubility issues were encountered, either 500 μL of DMSO or 500 μL of 1 M sodium hydroxide were used to dissolve the compounds prior to making the stock solution. A solution of DMSO (500 μL in 100 mL in PBS) was used as the void marker to determine the minimum retention volume (V_0 = 127.4 μL, s.d. = 2.3 μL) of the column. Blank experiments (*i.e.* a column packed with silica only) showed no non-specific interaction for any of the analytes.

Determination of binding constants (K_d) and the amount of HSA immobilised on the column (B_t) were performed using: $1/\Delta V = 1/B_t[A_0] + K_d/B_t$; K_d and B_t were obtained by plotting $1/\Delta V = f([A_0])$, ΔV being the difference between the retention volume of the injected compound (V) minus the retention volume of the void marker (V_0) (*i.e.* breakthrough volume), and $[A_0]$ the total concentration of the injected compound.

Validation of the system: injection of diclofenac (35) and isoniazid (36). Solutions of diclofenac sodium salt (**35**) and isoniazid (**36**) at 62.5 μM were prepared in PBS and sequentially infused through the column containing HSA using the HPLC method described in the previous section. Diclofenac was monitored at 220 and 254 nm whereas isoniazid was followed at 220 and 262 nm. Retention volumes of 130 μL (s.d. = 3.5 μL) and 837.5 μL (s.d. = 2.8 μL) were respectively obtained for these two reference compounds.

Assessment of the HSA column with zolpidem (1): determination of B_t. The first compound assessed using this assay was zolpidem (**1**). In practice, from a 125 μM stock solution of zolpidem (**1**) in PBS were prepared concentrations of 62.5, 41.67, 31.25, 15.62 and 7.81 μM. Each of these mixtures were then injected in triplicate following the same HPLC method, with B_t and K_d values being calculated as explained above. In these conditions, K_d and B_t were respectively found to be 61 μM and 20.7 nmoles. In order to show the reproducibility of this assay, the same experiment was performed after two months on the same column; B_t did not change over the two months period, validating the stability of the immobilised HSA on the column as well as the reproducibility of the technique.

Determination of the affinity of the synthesised compounds 1, 2, 15–34 for HSA. For each compound, stock solutions at 125 μM were prepared in PBS. 0.5% (vol) of either DMSO or 1 M sodium hydroxide were added when compounds were not completely soluble. Three concentrations (7.81, 31.25 and 62.5 μM, all within the linear range of the detector) were injected in triplicate and monitored simultaneously at two wavelengths following the same HPLC method with B_t and K_d values being calculated. An average B_t of 20 nmole was obtained throughout the experiments; K_d values are reported in Table 1.

Ranking method based on a single injection from aliquots automatically prepared from the reaction output. In FAC experiments, it is possible to rank compound affinities for the target molecule based on their retention volumes obtained *via* single injections. Although this will not produce B_t and K_d values, it is a quick way to compare relative affinities, especially when dealing with a large collection of compounds or/and dealing with outputs straight from flow chemistry reactors. In practice, 20 μL of the aliquot taken by the fraction collector was diluted further in 2 mL of PBS and injected directly using the standard HPLC method.

Compounds were simply ranked according to their retention volumes which correlated well with the trend seen in Table 1.

Acknowledgements

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