Brief Articles

Studies Directed at the Use of a Parallel Synthesis Matrix to Increase Throughput in an in Vivo Assay

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Heparin is the anticoagulant of choice for hospitalized patients, but it is dosed only by injection because it is not absorbed following oral administration. We have discovered and prepared compounds (delivery agents) that facilitate the gastrointestinal absorption of heparin in rats, monkeys, and humans when given orally. We are currently developing a parallel synthesis approach to increase our delivery agent screening throughput in vivo. This approach has been used to produce micromolar quantities of compounds for testing in rats in a 5 × 5 parallel synthesis array. Using an amine benzoylation reaction sequence, 10 mixtures were prepared. These mixtures contained equal weight quantities of five *N*-substituted, non-α, amino acid delivery agents. Each of these mixtures was orally administered to rats in combination with heparin, and plasma clotting times (APTT) were measured to determine activity. Deconvolution of the data accurately identified the most active individual components. Independent synthesis of these compounds verified their activity. This parallel synthesis approach is an effective tool for the screening of oral heparin delivery agents and has increased screening throughput significantly.

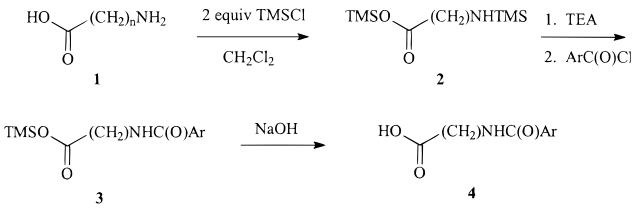
Introduction

Heparin is the anticoagulant of choice for hospitalized patients, but it is dosed only by injection because it is not absorbed following oral administration. We have discovered a series of compounds (delivery agents) that act to facilitate the oral delivery of heparin.[1,2]

As part of our continued efforts to develop an oral heparin, we routinely prepare and screen novel, oral, heparin delivery agent candidates. The candidate compounds are prepared individually on a micromolar scale and screened in vivo.[3] In these screening studies, a combination of one delivery agent and heparin is evaluated per study group. This traditional approach requires the preparation, purification, and structural identification of each delivery agent individually, and each in vivo study provides data on only one delivery agent.

To improve the throughput in these studies, a parallel synthesis approach was investigated. Parallel synthesis has become a successful tool for lead generation in the field of medicinal chemistry.[4] In general this technique is most valuable as a method of preparation of large numbers of compounds in nanomolar quantities for evaluation in in vitro receptor binding assays. To date, however, parallel synthesis has received little attention for studies in which micromolar quantities of material are needed for in vivo screening studies. This is most likely due to the fact that animal studies are inherently more variable than in vitro assays. As a result, interpretation of the data can be more difficult. In this particular case, the proposed studies were further complicated by the variability of the APTT assay.[8] Despite these challenges, we have been able to demonstrate the use of a 5 × 5 parallel synthesis matrix to improve the throughput of our in vivo oral heparin delivery agent screen that requires about 500 mg of material. The delivery agents tested in this matrix were selected based on our previous work.[3] This parallel synthesis approach allows the screening of 25 delivery agent candidates in 10 in vivo studies. The throughput rate is significantly greater than the traditional approach of screening 25 delivery agents in 25 studies.

Scheme 1. Synthetic Scheme for the Preparation of Delivery Agent Mixtures, M1−M10



Chemistry

We have previously reported on a high-yield amine benzoylation reaction sequence for the preparation of *N*-substituted amino acids.[6] This clean, high-yielding reaction appeared to be a good candidate for use in a parallel synthesis format.[7]

A dichloromethane solution of five amino acids (4-aminobutyric acid, 6-aminocaproic acid, 8-aminocaprylic

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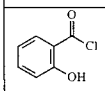
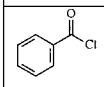
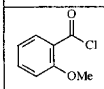
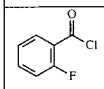
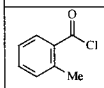
Scheme 2. Parallel Synthesis Matrix Starting Materials and in Vivo Data[a]

	4-amino butyric acid (APTT)	6-amino caproic acid (APTT)	8-amino caprylic acid (APTT)	10-amino decanoic acid (APTT)	12-amino docecanoic acid (APTT)	Mixtures (APTT)
	Cmpd **5** (31 ± 5)	Cmpd **6** (24 ± 2)	Cmpd **7** (125 ± 24)	Cmpd **8** (153 ± 50)	Cmpd **9** (86 ± 23)	**M6** (289 ± 59) ++
	Cmpd **10** (22 ± 1)	Cmpd **11** (33 ± 3)	Cmpd **12** (21 ± 1)	Cmpd **13** (31 ± 5)	Cmpd **14** (24 ± 3)	**M7** (150 ± 41) +
	Cmpd **15** (26 ± 4)	Cmpd **16** (31 ± 10)	Cmpd **17** (27 ± 2)	Cmpd **18** (50 ± 14)	Cmpd **19** (54 ± 8)	**M8** (68 ± 20) -
	Cmpd **20** (22 ± 1)	Cmpd **21** (28 ± 3)	Cmpd **22** (25 ± 2)	Cmpd **23** (27 ± 2)	Cmpd **24** (33 ± 5)	**M9** (45 ± 4) -
	Cmpd **25** (24 ± 2)	Cmpd **26** (26 ± 2)	Cmpd **27** (23 ± 1)	Cmpd **28** (34 ± 3)	Cmpd **29** (30 ± 2)	**M10** (88 ± 17) -
Mixtures (APTT)	**M1** (34 ± 1) -	**M2** (35 ± 6) -	**M3** (168 ± 57) ++	**M4** (242 ± 53) ++	**M5** (128 ± 22) +	

[a] The numbers in parentheses report the mean peak APTT (±SEM) in seconds for each mixture and individual compound following a single, colonic dose of the test compound in combination with heparin in rats.

acid, 10-aminodecanoic acid, and 12-aminododecanoic acid) was divided into five equal portions, and each portion was reacted according to Scheme 1 with one of the following five acid chlorides: acetylsalicyloyl chloride, benzoyl chloride, 2-anisoyl chloride, 2-fluorobenzoyl chloride, and 2-toluyl chloride. Conversely, a dichloromethane solution of these acid chlorides was divided into five portions and each portion was reacted according to Scheme 1 with one of the five amino acids. The resultant matrix is shown in Scheme 2. By design, each reaction product contained five compounds in equal amounts by weight. The mixtures were labeled M1−M10 (Scheme 2). The components of each mixture in this matrix were verified by independent synthesis and co-injection on a high-performance liquid chromatograph. Each of these mixtures essentially contained the five desired compounds as the only components and was used without further purification. Figure 1 shows a representative HPLC profile of a five-component mixture (M8) prepared by this chemistry.

Results and Discussion

Each compound mixture was tested colonically in rats for its ability to facilitate the gastrointestinal absorption of heparin. We have previously shown that colonic drug absorption is a good model for oral drug absorption.[8] Groups of five male Sprague−Dawley rats were administered by gavage a single, intracolonic (IC) dose of the delivery agent mixture in combination with heparin. Each experiment contained four dose groups. Blood samples were collected prior to dosing and at 0.25, 0.5, 1.0, and 1.5 h after dosing. Evidence of heparin delivery was indicated by an increase in blood clotting time measured by activated partial thromboplastin time (APTT).[5] A delivery agent mixture was considered to be active if a mean peak APTT level of >30 s was obtained.

Figure 2a shows the pharmacodynamic profile obtained following a single, oral dose of mixtures M1−M5 in combination with heparin. Figure 2b shows the pharmacodynamic profile obtained following administration of mixtures M6−M10 in combination with heparin. Scheme 2 reports the mean peak APTT (±SEM) values obtained in these studies. The data indicate that the three most active delivery agent mixtures are M3, M4, and M6. Of these, the two most active delivery agent mixtures are M4 and M6 with mean peak APTT levels of 242 ± 53 and 289 ± 59 s, respectively. The only delivery agent common to both of these mixtures is **8** suggesting that **8** should be the most active oral heparin delivery agent of the 25 compounds contained in the matrix. Delivery agent mixture M3 also shows good activity for oral heparin delivery producing a mean peak APTT level of 168 ± 57 s. No compounds are common to M3 and M4; however, **7** is contained in mixtures M3 and M6. Thus the data suggests that **7** should be a good oral delivery agent for heparin and be slightly less active than **8**. M7 and M5 also exhibit reasonable activity. Using the logic described above to analyze M3, M4, and M6 data, compounds **9** and **12**−**14** would also be expected to be effective oral heparin delivery agents.

To evaluate the ability of mixture testing to identify effective heparin delivery agents, the 25 compounds defined in the matrix were then each individually prepared and tested in rats for their ability to facilitate colonic heparin absorption. Some of these compound had

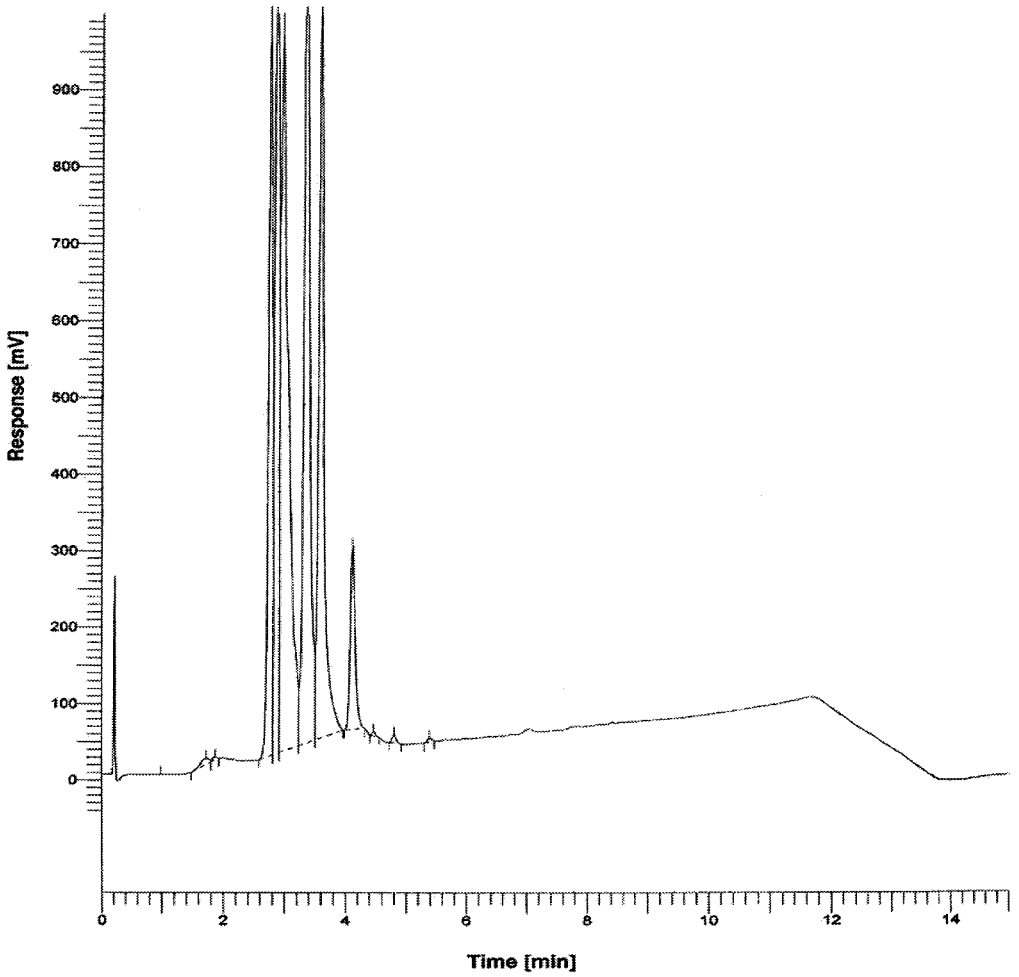


Figure 1. Representative reverse-phase HPLC of a five-component mixture.

been previously prepared and tested.[3] The data obtained from these studies is reported as mean peak APTT (Figure 3) and shows that compounds **7** and **8** are the most effective oral delivery agents for heparin. Compound **9** also has good activity when tested with heparin, and compounds **12**−**14** are significantly less active. It is interesting that mixture M7 shows reasonable activity; however, testing of its individual components (**10**−**14**) indicates that they are less active than expected. This observation can be explained by the fact that delivery agent activity is dependent on a variety of factors. Among these, aqueous solubility is important. Each of the compounds in mixture M7 has limited aqueous solubility under the conditions used to dose the discrete delivery agent/heparin combinations (50 mg/mL delivery agent). Thus, compounds **10**−**14** appear to have poor activity when tested individually. When these compounds are administered as a mixture, the concentration of each compound in the dosing solution is reduced considerably (10 mg/mL) and each component is soluble. Also, the heparin dose in the mixture dosing solution is increased. These two factors can be used to explain the apparent activity of M7. Thus, the matrix screening approach has correctly identified the three most active compounds in this group of 25 potential delivery agents in only 10 in vivo studies.

Conclusions

The use of a 5 × 5 parallel synthesis matrix to prepare mixtures of compounds for testing as oral heparin delivery agents has increased screening throughput significantly in this in vivo assay. This methodology allows the testing of 25 compounds in 10 experiments, while the traditional medicinal chemistry approach requires 25 experiments to test 25 compounds. Both the parallel synthesis and the traditional medicinal chemistry studies identified the same three delivery agents as the most active compounds and agreed the order of decreasing activity to be **8** > **7** > **9**.

Experimental Section

Chemistry. NMR spectra were recorded at 300 MHz in either D_2O or DMSO-d_6. Combustion analyses were performed by Microlit Laboratories, Madison, NJ, and were within acceptable limits (C, H, N ±0.4%). Thin-layer chromatography (TLC) was performed using E. Merck Kieselgel 60 F-254 plates. Reactions were monitored by high-pressure liquid chromotography (HPLC) on a Vydac 25- × 4.6-mm C_{18} protein and peptide column using a 0−50% gradient of acetonitrile in water with 0.1% trifluoroacetic acid. Melting points were performed using a Mel-Temp II from Laboratory Devices. All chemicals used in the syntheses of mixtures **1**−**10** and compounds **5**−**29** were purchased from Aldrich Chemical Co., St. Louis, MO, and prepared as described previously.[3]

General Procedure for the Preparation of Delivery Agent Mixtures. Preparation of M8. A mixture of 4-aminobutyric acid (434 mg, 4.2 mmol), 6-aminocaproic acid (495 mg, 3.8 mmol), 8-aminocaprylic acid (543 mg, 3.5 mmol), 10-aminodecanoic acid (582 mg, 3.1 mmol), and 12-aminodecanoic acid (616 mg, 2.2 mmol) was dissolved in methylene chloride (100 mL). Trimethylsilyl chloride (4.42 mL, 34.8 mmol) was added in one portion and the resulting mixture was heated to

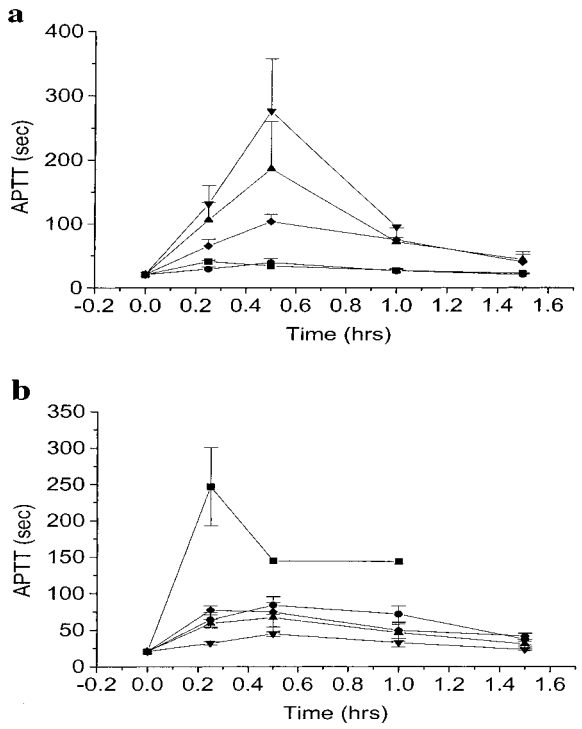


Figure 2. (a) Pharmacodynamic response in rats following a single, colonic dose of a delivery agent mixture (50 mg/kg) in combination with heparin (50 mg/kg): squares, M1; circles, M2; up-triangles, M3; down-triangles, M4; diamonds, M5. (b) Pharmacodynamic response in rats following a single, colonic dose of a delivery agent mixture (50 mg/kg) in combination with heparin (50 mg/kg): squares, M6; circles, M7; up-triangles, M8; down-triangles, M9;diamonds, M10.

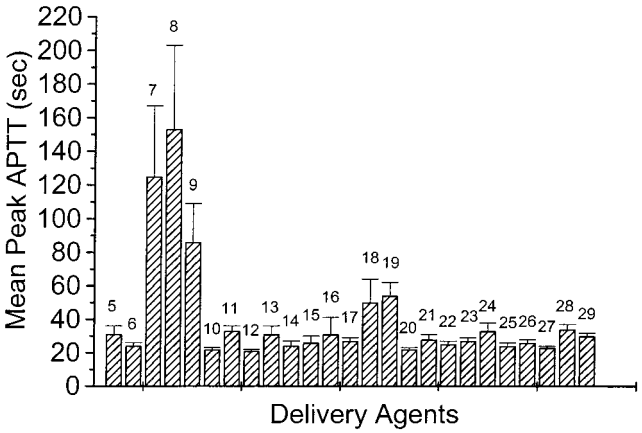


Figure 3. Mean peak APTT in rats following a single, colonic dose of a selected delivery agent (50 mg/kg) in combination with heparin (25 mg/kg).

reflux for about 2 h. The reaction was cooled to room temperature and triethylamine (3.64 mL, 26.1 mmol) was added dropwise followed by anisoyl chloride (2.58 mL, 17.4 mmol). The reaction mixture was stirred at room temperature overnight and then concentrated in vacuo. The residue was stirred in saturated sodium carbonate and acidified with hydrochloric acid (2 N). The resulting aqueous solution was extracted with ether (2 × 100 mL), dried over sodium sulfate, filtered, and concentrated in vacuo to give the desired five-component mixture (4.31 g) that was used without further purification.

Dosing Solution Preparation. The delivery agent mixture (150 mg) and heparin (75 mg) were mixed by vortex as dry powders. This dry mixture was dissolved in 25% v/v aqueous propylene glycol, and the apparent pH was adjusted to about 7 with 2 N aqueous sodium hydroxide. The final volume was adjusted to 3.0 mL. The dosing solution was sonicated to produce a clear solution with an apparent pH of about 7.

In Vivo Studies. Male Sprague−Dawley rats, housed in the animal facility at Emisphere Technologies, Inc., were acclimated for a period of at least 5 days prior to dosing. The animals weighed 300−350 g and were fasted for 12 h before dosing. Groups of 5 rats were anesthetized with 44 mg/kg ketamine hydrochloride intramuscularly immediately prior to dosing. Each group was administered a single, colonic dose (1 mL/kg) of the delivery agent/heparin combination via a 7.5-cm, 8-fr Rusch catheter attached to a 1-mL syringe. The dosing catheter was inserted into the colon through the anus until the tube was no longer visible, and the dosing solution was expressed slowly into the colon. Citrated blood samples were collected serially by cardiac puncture at 0, 0.25, 0.5, 1.0, and 1.5 h, plasma was harvested, and APTT values were measured.

Supporting Information Available: Experimental data for compounds **5**−**29**. This material is available free of charge via the Internet at http://pubs.acs.org.

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