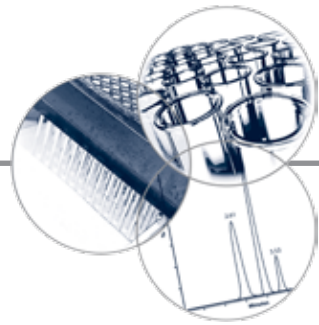

Bioanalytical determination of unstable endogenous small peptides: RFRP3 and its metabolites in rat blood

Background: Targeting the gonadotropin-releasing hormone pathway for the treatment of endometriosis leads to an interest in monitoring for endogenous modulators of this pathway (RFRP3 and kisspeptin) as baseline controls for treatment development. **Results:** Stabilization of RFRP3 was shown to be extremely difficult in a highly enzymatically active matrix, such as rat blood. Sample denaturing with solvent at collection was necessary due to enzyme inhibition being unsuccessful at stabilization leading to difficulties in sample processing. Monitoring multiple fragments formed in blood can aid in profiling these peptides once in-source conversion is controlled. **Conclusion:** generic high-sensitivity LC–MS/MS assay was developed for RFRP3 and the fragments formed from it in whole blood. Use of 2D chromatography circumvents concentration and retention issues related to small fragments with a normal flow setup, making a more open-access approach feasible.

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Gonadotropin-releasing hormone (GnRH) signaling has been shown to result in the pulsatile release of luteinizing hormone (LH) and follicle-stimulating hormone (FSH) [1]. This in turn leads to estradiol release, endometrium thickening and in some cases endometriosis [2]. GnRH super-agonists are the gold standard treatment for endometriosis, however complete blockade leads to ovarian suppression (infertility) and osteoporosis. Other possible ways of targeting the GnRH pathway are via the GPR54 or GPF147 receptors. Antagonism of the GPR54 receptor should result in blockage of LH and FSH release. The natural ligand to GPF54, kisspeptin, is a small endogenous **peptide** the addition of which should increase LH and FSH. RFamide-related peptide-3 (RFRP3) another endogenous signaling peptide is an agonist for the GPR147 receptor. Agonism at GPR147 should block LH secretion [3,4]. In order to test these hypotheses dosing these peptides and monitoring for their exposure whilst also monitoring the LH and FSH levels is desirable. Methodology for monitoring LH and FSH in plasma is widely available through standardized ELISA kits [5]; as such development of an assay for these hormones was not required, only methods for RFRP3 and kisspeptin were investigated. This article deals primarily with the challenges involved devising an LC–MS/MS method to monitor for RFRP3 in rat blood or plasma. These sorts of peptides are notoriously unstable in biological matrices [6]. As such, the challenges around stabilization of samples, determination of sites of instability and, in addition, the possibility to monitor for the resulting products of this instability are all covered herein. Methodology must be fast and robust to allow the developed assays to be widely used within an open-access environment. Whilst developing a method for RFRP3 was the main focus, additional attention was given to the fact that a similar assay for kisspetin would also be required. As such, the intent was to develop a methodology that could also be used to monitor for kisspeptin when required. Whenever challenges arose during development, the resolution that would be required for kisspeptin was also considered. Assays are available for kisspeptin, however the majority of these revolve around the assay of larger kisspeptins (namely kisspeptin 54), are mostly ELISA assays and are usually in human plasma rather than a much more enzymatically active rat blood [7]. We were aiming for a generic LC–MS/MS assay for much smaller signaling peptides (RFRP3: 968.5 amu; amino acid sequence: VPNLPQRF-NH2; and kisspeptin 10: 1302 amu; amino acid sequence: YNWNSFGLRF-NH2) so the existing methodologies were not appropriate.

Experimental

Materials

Control rat blood (stabilized with K_2EDTA or Lithium Heparin) was obtained from in-house stocks and stored in 10-ml Sarstedt tubes (VWR International, Leicestershire, UK). Control blood

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Key Terms

Peptide: Molecule constructed from a chain of amino acid base units.

N-terminus cleavage: Shortening of a peptide by separating whole amino acids from the molecule end that naturally terminates in an amide.

Enzymatic stabilization: Using inhibitors added to a matrix sample in order to prevent compound degradation via attack from common endogenous enzymes (e.g., proteases, esterases and amidases).

was stored at approximately 4°C when not in use and deemed viable for 2 weeks. All *in vivo* blood samples were collected into BD Microtainer tubes precoated with K_2EDTA (VWR international, Leicestershire, UK) containing methanol.

RFRP3 was purchased from Phoenix Pharmaceuticals (Burlingame CA, USA). Leuprolide was purchased from Bachem (Bubeddorg, Switzerland). All the **N-terminus cleaved** fragments (PNLPQRF-NH2 [heptamer], NLPQRF-NH2 [hexamer], LPQRF-NH2 [pentamer], PQRF-NH2 [tetramer], and kisspeptin 10 were made available through Pfizer Worldwide Research and Developement's internal chemical library. All solvents and mobile phases were either purchased from Romil (Cambridge, UK) or Sigma-Aldrich or were prepared in-house. All solvents were of HPLC grade. HPLC-grade water was produced in-house (Millipore, Watford, UK).

After receipt, all Wistar Hans Rats (Charles River Laboratories, UK) were allowed to acclimatize for a period of 5 days prior to dosing and sample collection. Rats were stored as small groups of less than ten animals with light, temperature and humidity being regulated. Free access to food and water was made available at all times. Animals were sacrificed by terminal anesthesia followed by cervical dislocation.

■ Stock solutions

RFRP3 solid (1 mg) was dissolved in 968 µl of DMSO to create a 1 mM stock solution. A 600 µl aliquot of this stock was then further diluted with 2400 µl of water to prepare a 200 µM working stock. Both were deemed viable for 3 months.

All the N-terminus cleaved fragments (heptamer, hexamer, pentamer and tetramer) were similarly dissolved in sufficient DMSO to generate a 1 mM solution before being further diluted in water to generate working stocks.

Table 1. Generic HPLC system for direct injecting onto a UHPLC C18 column†.

Time (min)	Flow (µl/min)	MPA (%)	MPB (%)
0	1000	100	0
0.1	1000	100	0
0.65	1000	0	100
1.65	1000	0	100
1.7	1000	100	0
2	1000	100	0

†UHPLC column was maintained at approximately 60°C. Eluting pump: MPA = 0.1% formic acid in water:methanol (9:1 v/v); MPB = 0.1% Formic acid in water:methanol (1:9 v/v); Column = Zorbax Eclipse XDB C18 3.0 x 50 mm, 1.8 µm.
MPA: Mobile phase A; MPB: Mobile phase B.

All DMSO stocks were stored at 5°C in glass containers and showed good stability over a period of 3 months [Data not shown].

■ Blood stability time courses

A blood stability time course in whole rat blood was prepared by spiking 10 µl of a 200 µM solution of RFRP3 into 2 ml of rat blood to generate a pooled sample at 1 µM. This pool was split in half with one half being stored at 37°C and the other half being stored at room temperature (RT). Sub-samples (50 µl) were taken from each pool at time points 0, 5, 10, 20, 30, 45, 60 and 120 min. At each time point one sample from the 37°C pool and one sample from the RT pool were taken, the samples were each individually dropped into tubes containing 500 µl of acetonitrile to stop all enzymatic activity. These tubes were then vortex mixed and centrifuged before 300 µl of the acetonitrile was transferred into a second tube for analysis. Once all the samples were taken, centrifuged and transferred, they were all diluted 1:1 with water before injection onto an LC–MS/MS system.

Once a full chromatographic system was in place to assay for RFRP3 (and all the derived fragments) the 2-h samples from this time course were re-assayed to determine which RFRP3 fragments were present and likely to be of most interest to monitor when assaying *in vivo* samples

Subsequently, an **enzymatic stabilization** time courses in blood was prepared as above, only in this case 4 pools were prepared [8]. Pool 1 had no stabilizer added, pool 2 had sodium fluoride added to the blood before spiking (2 mg/ml NaF in pooled blood), pool 3 had *bis*-(4-nitrophenyl) phosphate (BNPP) added as a stabilizer (0.1 mg/ml in pooled blood) and pool 4 had Roche complete protease inhibitor cocktail (one capsule dissolved as directed for spiking into the pooled blood). These pools were stored at RT and were sampled as above at 0, 5, 10, 20, 30 and 40 min. As above the samples were protein precipitated, vortexed, transferred and diluted before injection at each time point.

For the stability of blood directly sampled into solvent again one pool of blood was spiked at 1 µM and split in half. One half was stored at RT the second half was aliquoted (50 µl) into tubes containing 500 µl of acetonitrile. These tubes were then stored at RT. At each time point (0, 10, 20, 30, 45, 60 and 120 min) one sample was taken from the whole blood (50 µl) and dropped into 500 µl of acetonitrile and one of the aliquots stored in acetonitrile was taken. Both were

Table 2. Generic HPLC system for trapping onto a C18-based cartridge followed by back washing and analytical resolution on a UHPLC C18 column†.

	Eluting pump‡			Loading pump§			
Time (min)	Flow (µl/min)	MPA (%)	MPB (%)	Flow (µl/min)	MPA (%)	MPB (%)	Valve: trap in-line (Y/N)
0	1000	100	0	1000	100	0	N
0.5	1000	100	0	1000	100	0	Y
0.6	1000	100	0	1000	100	0	Y
0.7	1000	100	0	1500	0	100	Y
0.95	1000	0	100	1500	0	100	Y
1.2	1000	0	100	1500	0	100	N
1.8	1000	0	100	1500	0	100	N
1.85	1000	0	100	1500	100	0	N
1.9	1000	100	0	1500	100	0	N
2.5	1000	100	0	1000	100	0	N

†Both trap and column were maintained at approximately 60°C.
‡ MPA: 0.1% formic acid in water:methanol (9:1 v/v); MPB: 0.1% formic acid in water:methanol (1:9 v/v); Column: Zorbax Eclipse XDB C18 3.0 x 50 mm, 1.8 µm.
§MPA: 3mM NH_4OAc + 0.03% FA:MeOH (9:1 v/v) + 0.1% TFA; MPB: MeOH:IPA:acetone (4:3:3 v/v/v) + 1% AcOH; Trap: OPTI®TRAP EXP™ C18 4.6 x 5.0 mm.
MPA: Mobile phase A; MPB: Mobile phase B, NH4OAc: Ammonium acetate, FA: Formic acid, MeOH: Methanol, TFA: Trifluoroacetic acid, IPA: Propan-2-ol, AcOH: Acetic acid.

vortexed and centrifuged, then 300 µl was transferred to a second tube and diluted 1:1 with water then injected as above. This procedure was later repeated using methanol in place of acetonitrle.

■ MS optimization

Solutions of RFRP3 (VPNLPQRF-NH2) and its resulting N-terminus cleaved fragments (PNLPQRF-NH2, NLPQRF-NH2, LPQRF-NH2 and PQRF-NH2) were directly infused into an API4000. *m/z* transitions were tuned for all of these fragments looking into both the multiple and single charged species.

Attention was also given to investigating which (if any) of the fragments from RFRP-3 were generated in-source, as controlling this is important when assaying for the smaller fragments to give confidence that the fragment is generated from the sample not the mass spectrometer when no or partial chromatographic separation is possible.

Table 3. Resoluablization of peptides after evaporation under nitrogen. Recovery calculated as percentage mean response relative to initial stock mean response.

	Acidified methanol in water						
Initial stock	**40%**	**50%**	**60%**	**70%**	**80%**	**90%**	**100%**
RFRP3 peak area							
1,600,000	611,000	1,010,000	1,330,000	1,580,000	1,540,000	DnP	DnP
1,590,000	632,000	1,040,000	1,350,000	1,550,000	1,590,000	DnP	DnP
1,590,000	664,000	1,070,000	1,320,000	1,550,000	1,580,000	DnP	DnP
1,590,000	636,000	1,040,000	1,330,000	1,560,000	1,570,000	DnP	DnP
% recovery	**40.0%**	**65.4%**	**83.6%**	**98.1%**	**98.7%**		
Kisspeptin peak area							
1,010,000	DnP	DnP	210,000	315,000	495,000	537,000	561,000
1,030,000	DnP	DnP	253,000	303,000	477,000	446,000	592,000
1,090,000	DnP	DnP	255,000	301,000	424,000	501,000	487,000
1,040,000	DnP	DnP	239,000	306,000	465,000	495,000	547,000
% recovery			**23.0%**	**29.4%**	**44.7%**	**47.6%**	**52.6%**

DnP: Data not processed.

Key Terms

2D-HPLC–MS/MS: HPLC performed in two orthogonal dimensions before analysis by MS/MS.

Half-life: Amount of time taken for the available concentration of compound present to halve.

Having determined *m/z* transitions for each fragment and the parent, solutions of each individual compound were directly injected whilst simultaneously monitoring for all of the other fragments. This enabled determination of which (if any) of the compounds cross converted in-source leading to potential false positives for that fragment.

■ LC optimization

UHPLC–MS/MS analysis was performed using a CTC autosampler (Presearch, Hants, UK), HP1100 binary HPLC pump (Agilent Technologies, Cheshire, UK), HP1200RR binary UHPLC pump (Agilent Technologies, Cheshire), UK HP1200RR column oven with integral switching valve (Agilent Technologies, Cheshire, UK) and an Applied BioSystems API4000 triple quadrupole mass spectrometer operating a TurboIonSpray™ ionization source and running on Analyst version 1.4.2 (Applied Biosystems, Cheshire, UK).

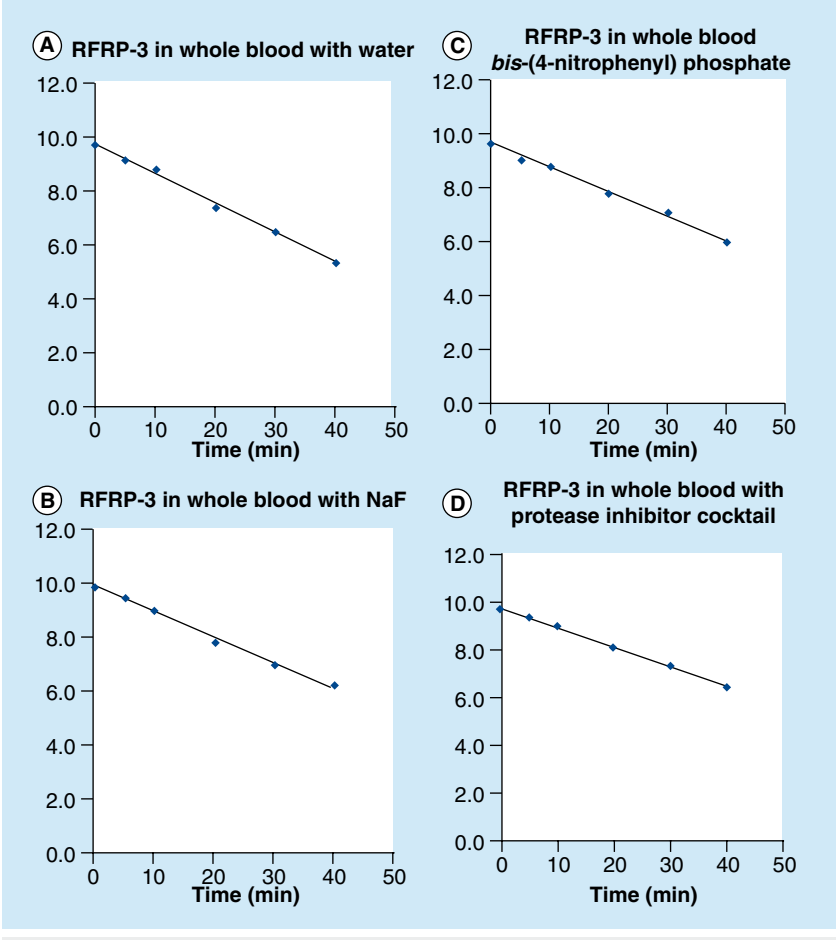

Figure 1. The loss of RFRP3 in blood when incubated with and without inhibitors. Half-lives are **(A)** water: 6.4 min, **(B)** NaF: 7.5 min, **(C)** BNPP: 7.7 mins and **(D)** protease inhibitor cocktail: 8.4 mim.

Initially samples were directly injected onto and analytically resolved on a Zorbax Eclipse XDB C18 3.0 × 50 mm, 1.8 µm (Agilent Technologies, Cheshire, UK) at a flow rate of 1.0 ml/min (full details of the direct injection HPLC method can be found in Table 1).

The final LC system setup was changed to run as a **2D-HPLC–MS/MS** system using ion-paired, C18 retention followed by straight C18 chromatography. The samples (after injection) were initially loaded onto an OPTI®TRAP EXP™ C18 4.6 × 5.0 mm trap cartridge packed with halo C18 material (ACR Sciences, Hampshire, UK) at a flow rate of 1.0 ml/min using a loading mobile phase of 10% methanol containing 0.1% TFA, after washing the trapping cartridge with loading mobile phase, the sample was then back eluted and analytically resolved on a Zorbax Eclipse XDB C18 3.0 × 50 mm, 1.8 µl column (Agilent Technologies, Cheshire, UK) at a flow rate of 1.0 ml/min (full details of the 2D LC HPLC method can be found in Table 2).

Method validation & calibration preparation

Having determined the most appropriate method of stabilization, the most important fragments to monitor and an LC setup to monitor them, a test batch of samples were prepared to validate the appropriateness of this assay to be used for *in vivo* samples.

A test batch was prepared consisting of calibration standards (of RFRP3, hexamer and pentamer) spiked at 1, 2, 5, 10, 20, 50, 100, 200, 500 and 1000 ng/ml (~1–1000 nM) QC samples at 5, 50 and 500 ng/ml a blank blood sample and at least one blank blood sample containing IS. In addition, this batch contained triplicate QC samples spiked at 50 ng/ml, triplicate blank blood samples extracted then over spiked with RFRP3 at a representative level to be equivalent to a 50-ng/ml sample and triplicate solutions in solvent of RFRP3 at an equivalent concentration to a 50-ng/ml sample to assess recovery and matrix effects.

Owing to the extremely short **half-life** of RFRP3 in whole blood, great care had to be taken in the preparation of the calibration and QC samples. As such, spiking solutions were prepared at 10-times the concentration required in 25% methanol/water (v/v). Then for each calibration standard (and QC sample), 45 µl of pooled blank blood was pipetted into a tube, then 5 µl of the appropriate spiking solution

was added and the blood very briefly vortexed. A 500-µl aliquot of acetonitrile was then immediately added to the tube, which was vortexed again to stop all the enzymatic activity. This process was completed for each individual standard before commencing the preparation of the next sample.

Once all the samples had been prepared they were all centrifuged and 150 µl of the acetonitrile was transferred to a 96-well plate where 150 µl of water was added to each sample (containing leuprolide IS). For the matrix effect samples, the precipitated blank whole-blood samples had water added that contained RFRP3 at 4.54 ng/ml to give the appropriate final concentration. For the solvent samples, 150 µl of clean acetonitrile had the same water containing 4.54 ng/ml of RFRP3 added.

To confirm stability, the QC samples were all prepared in duplicate and after initial sampling of one set was left at -20°C overnight and the second set was left at RT overnight. These samples were re-assayed the next day against a freshly prepared calibration line that was prepared as detailed above.

For the second validation batch, samples were prepared as noted above, substituting methanol for the acetonitrile used to precipitate the blood.

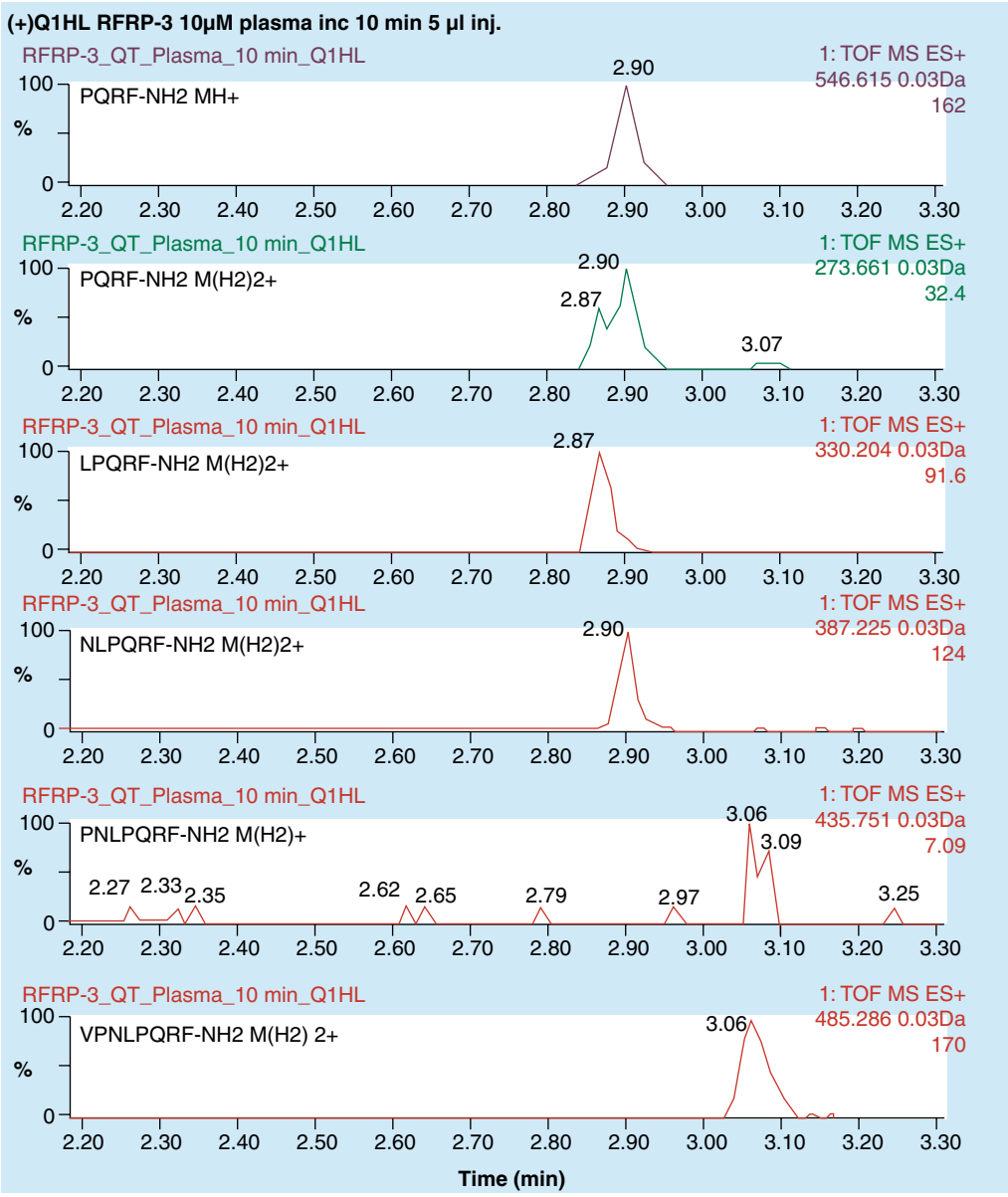

Figure 2. Peak response of N-terminus cleaved fragments present in incubated blood containing only RFRP3.

Again QC samples were left at -20°C and at RT overnight the re-assayed the next day with a fresh calibration line.

■ *In vivo* intravenous infusion

Having semi-validated an appropriate method for sample assay, an experiment to monitor for these peptides *in-vivo* was undertaken.

A dose-escalating intravenous infusion study was performed to determine whether we could achieve measurable levels of RFRP3 and to monitor the rate at which the N-terminus cleaved fragments are formed.

The aim of this study was to profile the pharmacokinetics of RFRP3 following intravenous infusion of 0.3 mg/kg over 30 min, followed by 1 mg/kg over the next 30 min and 3 mg/kg over the next 30 min in rats. A solution of 0.6 mg/ml of RFRP3 in 10% DMSO in saline was prepared for infusion. Infusion rates were 500 µl/1800 sec/kg for 30 min (1 ml/h/kg); 1670 µl/1800 s/kg for 30 min (3.3 ml/h/kg); 5000 µl/1800 s/kg for 30 min (10 ml/h/kg). Total infusion rate was 7.2 ml/kg over 1.5 h.

Blood samples (25 µl) were taken via an *in situ* cannula into EDTA tubes containing 250 µl methanol using a Dilab Accusampler at time intervals of predose, 1, 5, 10, 15, 20, 30, 31, 35, 40, 45, 50, 60, 61, 65, 70, 75, 80, 90, 91, 95, 100, 105, 110 and 120 min. Each sample was vortexed immediately on collection and stored deep frozen (-20°C) until analysis.

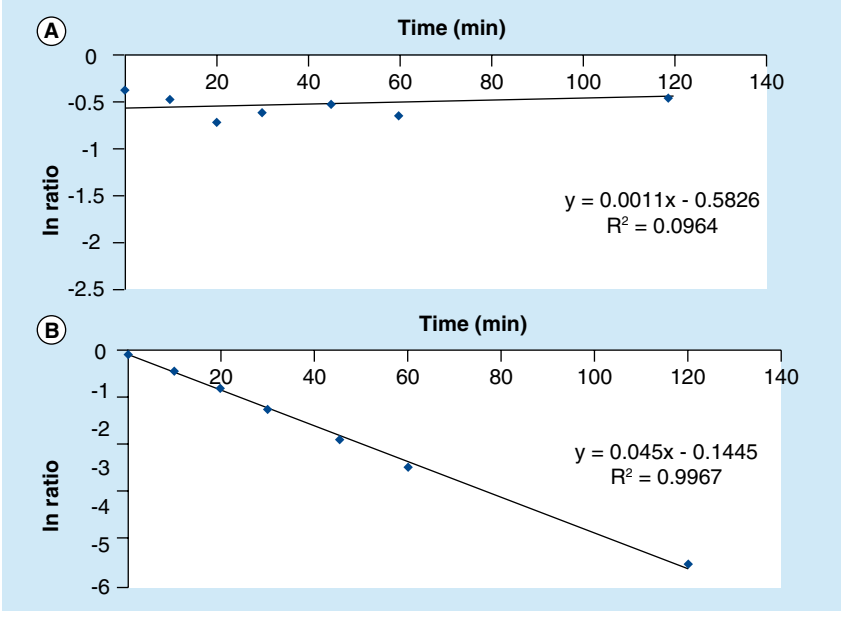

Figure 3. Loss of RFRP3 in blood stored (A) with and (B) without acetonitrile denaturing.

Having reviewed the *in vitro* data and likely low levels expected in future *in vivo* experiments the calibration ranges used for *in vivo* data were 0.1–500 ng/ml for RFRP3 (~100 pM–500 nM), and 1–5000 ng/ml for all the fragments. Calibrants and QC samples were prepared as described in the method validation section. *In vivo* study samples were received frozen in tubes containing the blood in methanol. All *in vivo* samples were thawed at RT before being vortex mixed then centrifuged. A 150-µl aliquot of the methanol was then transferred to appropriate wells of a 96-well plate, which had previously had 150-µl aliquots of methanol transferred from the freshly prepared calibration and QC samples where appropriate. This plate was then diluted 1:1 with water containing leuprolide IS for assay. We injected 250 µl of these samples into the semi-2D-LC system described earlier.

Results & discussion

■ Blood stability time courses

Basic compound stability of the parent peptide was tested in acid/base aqueous and solvent before being injected on a generic direct injection LC system to check for any loss of signal from storage. In addition, samples of RFRP3 were prepared in solvent before being dried down under nitrogen and reconstituted to check for any signal loss during post-extraction sample concentration. No degradation of RFRP3 was observed in any combination of acid, base, solvent or aqueous giving confidence for continued testing of actual stability of the peptide within the biological matrix. However, when RFRP3 in solvent was dried down and reconstituted, as expected, there was difficulty in getting the peptide back into solution. This re-solubilization was attempted in both glass and polypropylene. In both cases re-suspension of RFRP3 (post dry-down) required a very high percentage of organic modifier in the solvent. Close to 100% re-suspension was achieved at approximately 70% organic modifier. As we were intending to subsequently use this same methodology for assaying kisspeptin 10, solutions of kisspeptin 10 were also prepared in solvent dried down and reconstituted and in this case after attempting solvents all the way from fully aqueous to fully organic the best recovery possible was in the region of 50% (Table 3). This led to a need to try and develop a method that did not require sample dry down for postextraction concentration.

The initial time course assay of RFRP3 spiked into whole rat blood then sub-sampled over a period of 2 h showed a very rapid decline in the available concentration of RFRP3 confirming our suspicion of it being very unstable in an aggresive biological matrix such as rat blood (this experiment was repeated as a control in the stabilization experiment shown in Figure 1). These samples were subsequently injected on an accurate mass time of flight MS (Waters Q-ToF Premier, Elstree, Hertfordshire, UK) scanning Q1 for the *m/z* of the whole RFRP and all the smaller peptides created by a single amino acid cleavage at the N-terminus Figure 2. This showed that some of these peptide fragments were present at the latter time points, as distinct peaks are separated from RFRP-3. The heptamer appeared as a small peak co-eluting with RFRP3, and was disregarded as a likely dominant product. There was a large peak of the hexamer separated from the RFRP3 peak and this was also deemed one of the likely dominant fragments. Finally, there was a peak for the pentamer that was chromatographically separated, as such this was taken as the last likely dominant fragment. There were peaks for the tetramer but all these peaks were co-eluted with either the pentamer or the hexamer and as such, were most likely in-source fragmentation. The tetramer was also disregarded as a likely dominant fragment.

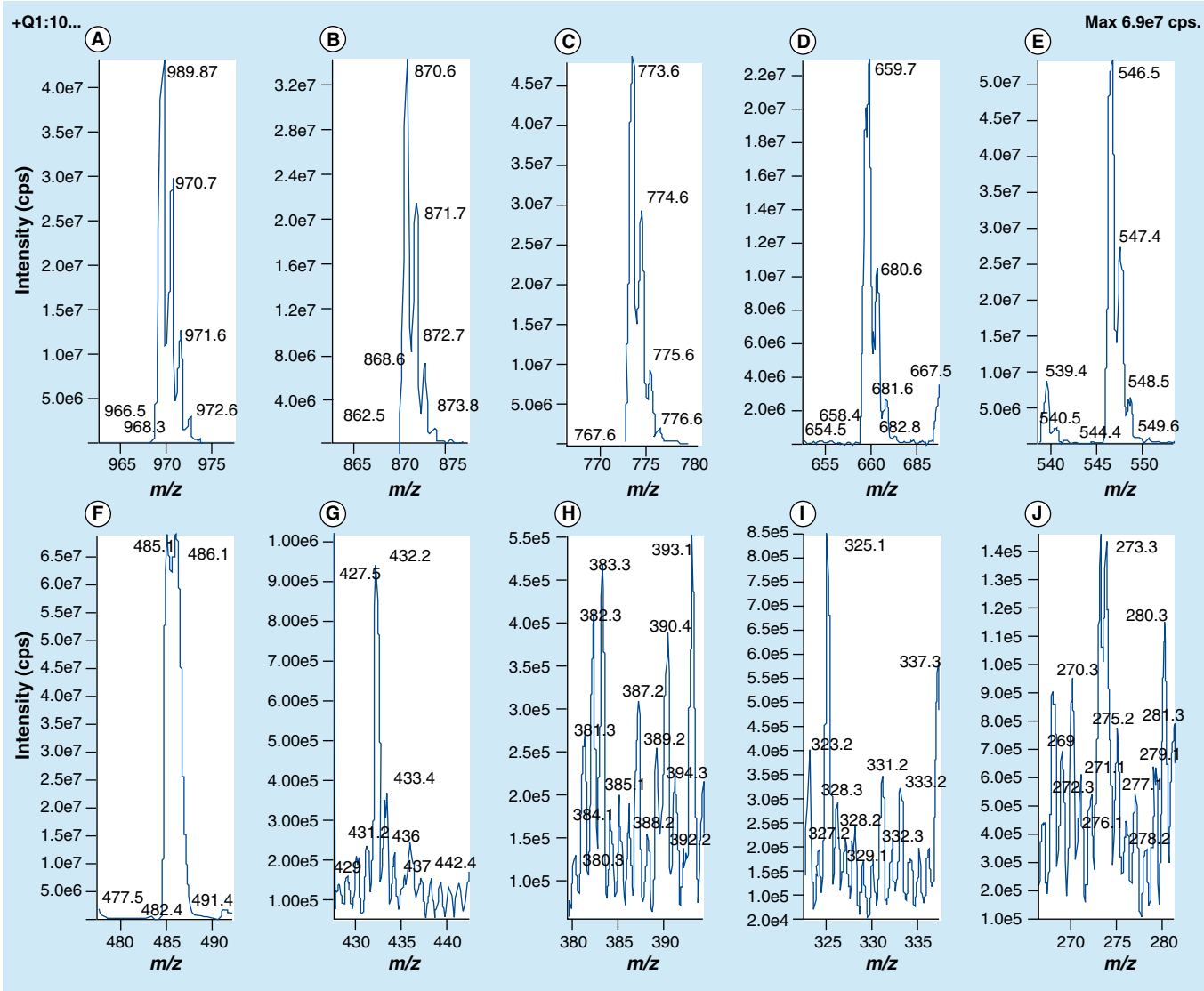

Figure 4. Q1 scan monitoring for the single- and double-charged species of RFRP3 and fragments whilst only infusing RFRP3 in solution. (A) RFRP3 [M+H]+ *m/z* 970, **(B)** heptamer [M+H]+ *m/z* 870, **(C)** heptamer [M+H]+ *m/z* 774, **(D)** pentamer [M+H]+ *m/z* 660, **(E)** tetramer [M+H]+ *m/z* 546.5, **(F)** RFRP3 [M+2H]2+ *m/z* 485.5, **(G)** heptamer [M+2H]2+ *m/z* 435.5, **(H)** heptamer [M+2H]2+ *m/z* 387.5 **(I)** pentamer [M+2H]2+ *m/z* 330.3 and **(J)** tetramer [M+2H]2+ *m/z* 273.7.

Whilst having selected RFRP3, the hexamer and the pentamer as the most important peptides to monitor, we continued in our attempt to produce a method capable of monitoring all fragments. This was for confirmation that the fragmentation pattern generated from *in vitro* samples was duplicated across the *in vivo* samples.

The stability timelines for RFRP3 in whole rat blood containing enzymatic stabilizers were run using our generic gradient, every stabilizing agent still showed massive degradation of the whole RFRP3 (Figure 1). In the worst case (no inhibitors) a 6.4-min half-life was determined; in the best case, using the full protease inhibitor cocktail, the half-life only increases to 8.4 min. This led to the only plausible stabilization available, which was to directly sample the blood into a solvent to denature it and prevent any further enzymatic digestion. Direct denaturing was a likely methodology to follow as we had observed that all samples, once protein precipitated for injection, remained stable enough for reinjection the next day (if required).

There are multiple references to using protease inhibitors to stabilize peptides such as these in human plasma. However, often these are quoted for larger peptide bodies (i.e., kisspeptin 54) [9], whereas we were looking to stabilize the smaller fragments RFRP3 and kisspeptin 10. In addition, stabilization is usually cited in human plasma whereas we were looking to try stabilization in much more enzymatically active rat blood. Some degrees of stabilization were seen when using very high concentrations of aprotinin in plasma, however, even at these concentrations we found that the stabilization in whole blood was still negligible.

The time course that was prepared to compare acetonitrile precipitated samples against untreated whole-blood samples was assayed whilst simultaneously monitoring for both RFRP3 and hexamer (Figure 3). By looking at both the RFRP3 concentrations and the ratio of hexamer to RFRP3 in all samples, we were confident that this procedure would report accurate results for blood concentrations at the time of sampling.

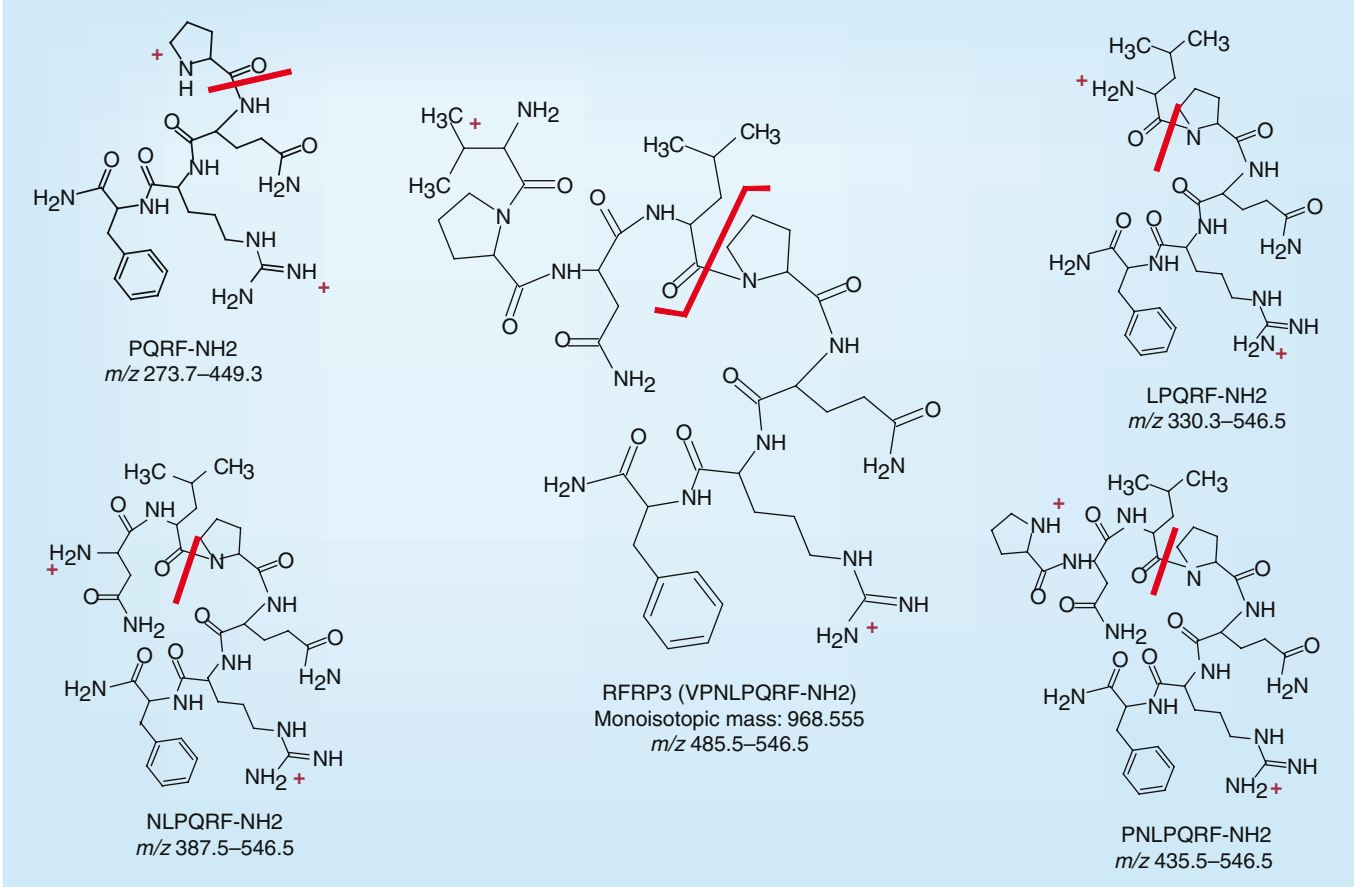

Figure 5. Structures, transitions and fragmentations of RFRP3 and its N-terminus fragments.

MS optimization

The infusion of RFRP3 directly into the MS source did show in-source fragmentation leading to the appearance of *m/z* charge peaks corresponding to heptamer, hexamer, pentamer and tetramer whilst only infusing RFRP3 (FIGURE 4).

When tuning all the fragments, and RFRP3 itself, it was possible to tune each as either a double- or a single-charged parent. When reviewing chromatograms of samples monitoring the single-charged parent transition there was a great deal of in-source fragmentation leading to peaks appearing for the smaller fragments underneath the peak for the parent compound. However, when the same samples were re-tested using the double-charged transitions, virtually all incidents of in-source cross-talk were eliminated (a slight cross-talk for pentamer to tetramer was still noted). With regard to sensitivity the double-charged transitions gave increased sensitivity for the larger peptides but reduced sensitivity for the smaller fragments. This reduced sensitivity at the smaller fragments end was an acceptable price to pay for the improvements in specificity it brought. However, as complete chromatographic separation was possible for the smallest peptides, it would be possible to monitor them using the single charged ions if increased sensitivity was required.

The final MS parameters selected for use in all assays were as follows:

- Generic MS parameters: collision activated dissociation gas (6.00), curtain gas (20.0), nebulizer gas (50.0), heater gas (50.0), temperature (700), ion-spray voltage (5500), declustering potential (60.0) and entrance potential (10.0).
- Multiple reaction monitoring (MRM) ion transitions and other compound specific parameters: RFRP3 (*m/z* 485.5–546.5, collision energy [CE] [30], collision exit potential [CXP] [16]), NLPQRF-NH2 (*m/z* 387.4–546.5, CE [18], CXP [16]), LPQFR-NH2 (*m/z* 330.3–546.5, CE [20], CXP [17]), Leuprolide (IS) (*m/z* 605–249, CE [40], CXP [7]), In all cases the dwell time was set at 25 msec per MRM ion transition (structures and fragmentation; FIGURE 5).

LC optimization

Initial sample assays run on the generic direct injection system gave good retention and sensitivity for RFRP3. Unfortunately, there were

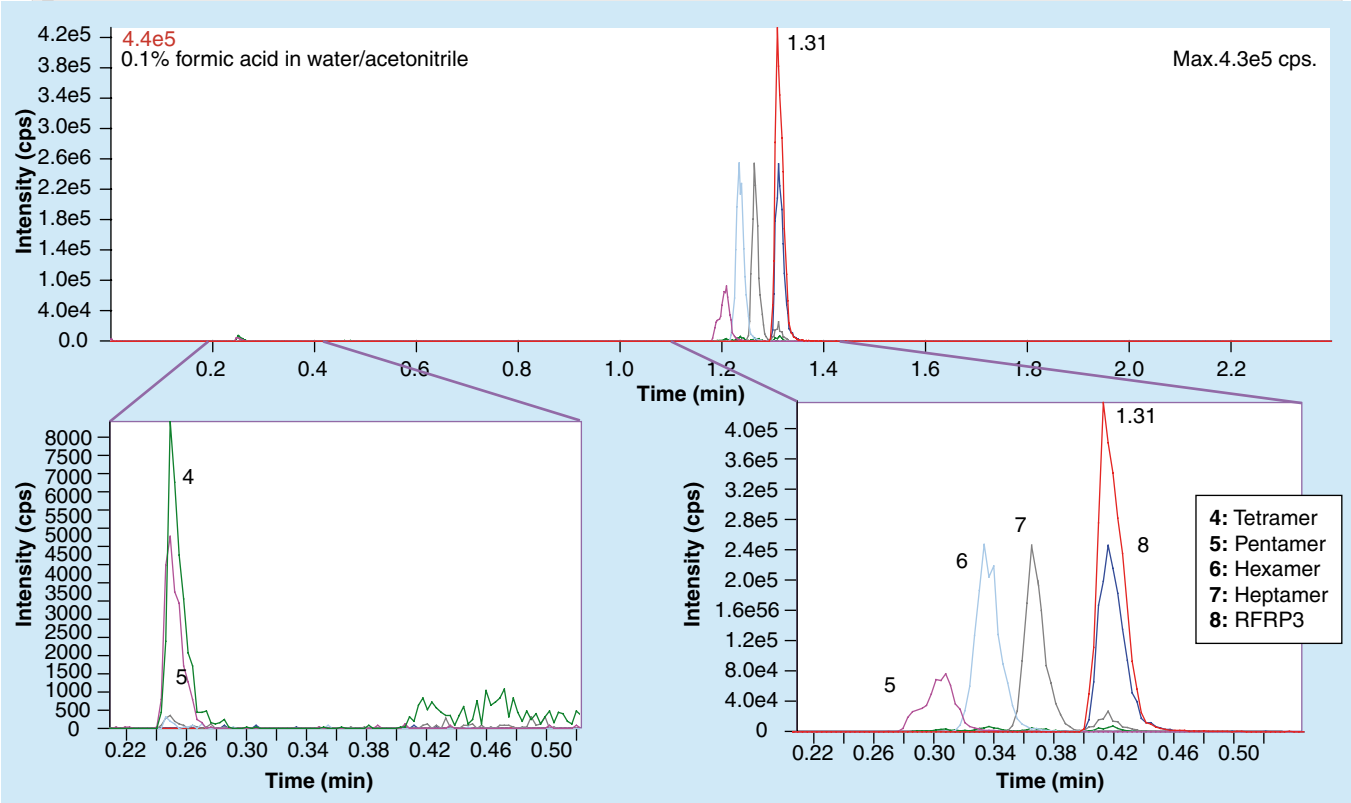

Figure 6. Chromatogram monitoring. Sample run in direct injection with simple 0.1% formic acid mobile phase. Raw sensitivity of RFRP3 4.4e5.

complications that arose with this approach. Whilst attempting to simultaneously monitor for all the smaller fragments (tetramer, pentamer, hexamer and heptamer) at the same time as RFRP3, we found that the tetramer and pentamer were, at best, only partially retained. The majority, if not all, of these smaller fragments were eluted at the solvent front and suffered massive suppression (Figure 6). In addition with the sample having to be directly dropped into solvent at collection for stability, we had very limited sample pretreatment and there was evidence of suppression on the peaks of interest from endogenous material when directly injecting the precipitated blood. With direct sample denaturing at collection we have a large predilution of the peptide into solvent. When attempting to dry down this solvent and reconstitute to concentrate the sample back up, getting the peptide back into solution proved to be a challenge. As such, we pursued the ability to inject large volumes to alleviate the need for sample concentration. Some success was obtained by introducing TFA into the mobile phase as this did give retention for all the fragments, however, this did result in both losing a degree of the separation we had running without TFA and also introducing a sizable suppression of signal at the MS (Figure 7) [10,11]. All of these issues were resolved by switching to the semi-2D-LC setup previously detailed. With this set up we were able to both prewash the sample on the loading column whilst also preconcentrating it to get fully linear loading volumes even when back flushing onto the smaller diameter columns required for sensitivity. Also in this step we had the ability to load with TFA present in the mobile phase at a high enough concentration that the smaller, more polar, fragments of RFRP3 (the tetramer and pentamer) remain retained on the second column. However as the second column mobile phase contained no TFA, we did not see the suppression of signal normally associated with TFA based mobile phases (Figure 8).

By using this setup we were able to retain required sensitivity levels of RFRP3 (with no notable suppression) whilst, in addition, retaining the smaller RFRP3 fragments of interest and retaining sufficient separation of peaks for specificity where required (pentamer to tetramer in-source conversion). Although the hexamer and pentamer were not fully resolved in this chromatography there was no notable conversion observed in-source between them when monitoring both on a double-charged parent. In addition, with the

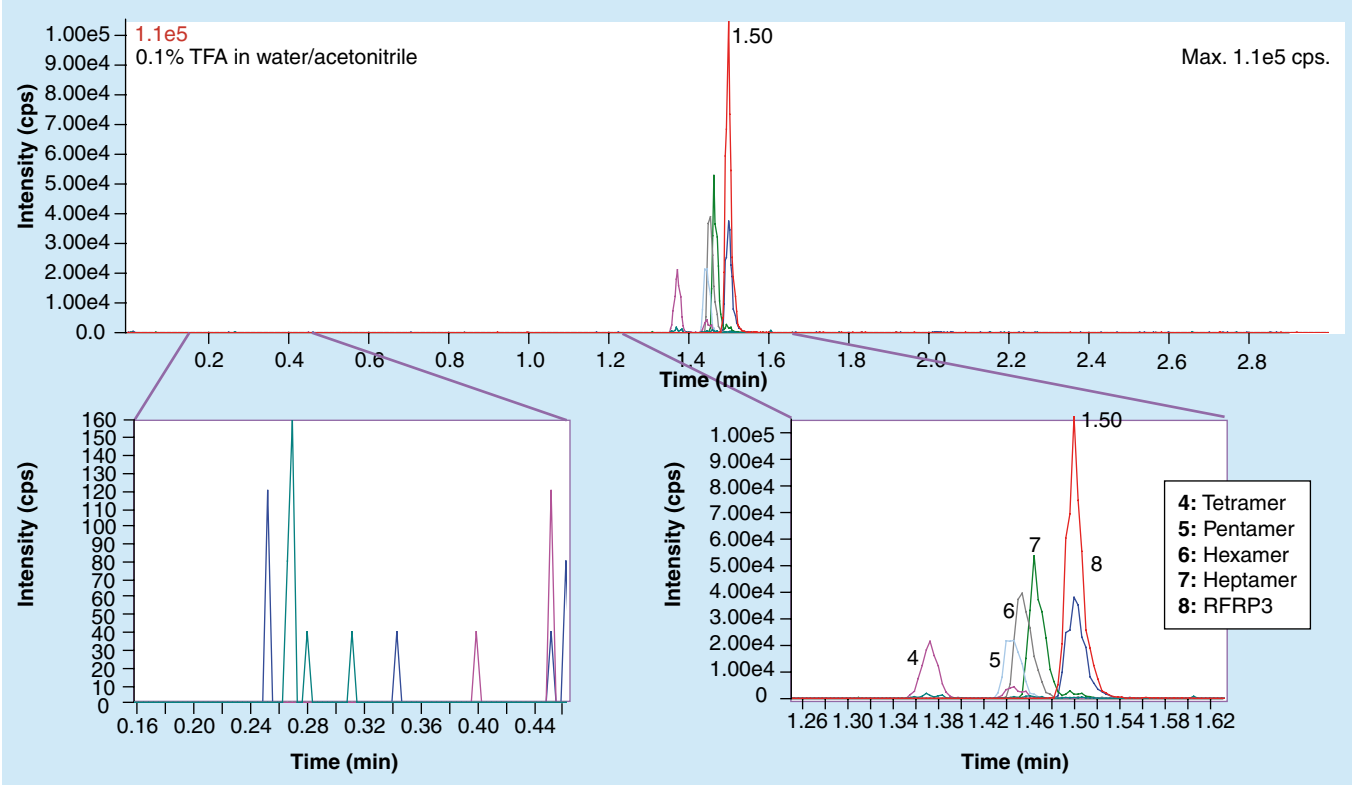

Figure 7. Chromatogram monitoring. Sample run in direct injection with 0.1% TFA added as ion-pairing agent in mobile phase. Raw sensitivity of RFRP3 1.1e5.

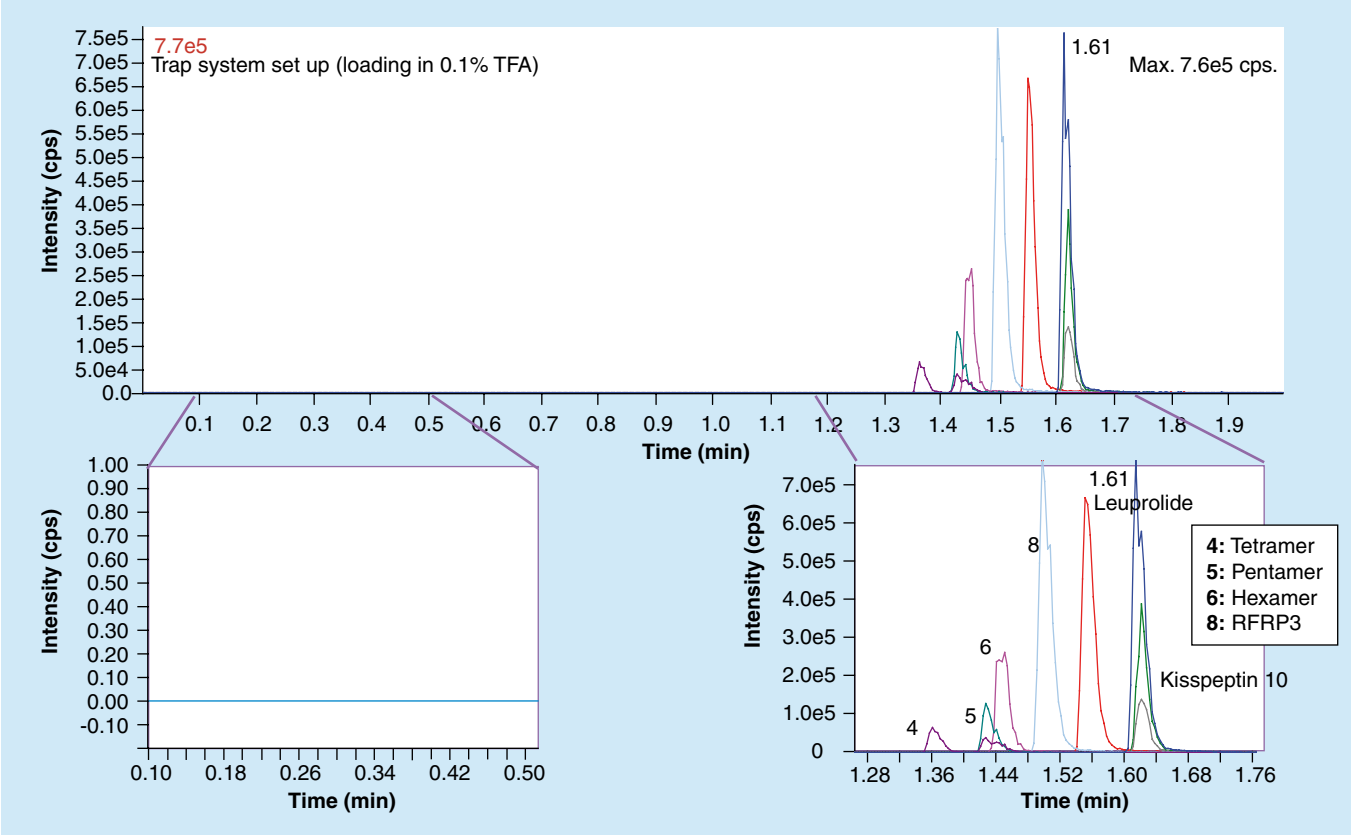

Figure 8. Chromatogram monitoring. Sample run Using trap and elute with ion pairing in loading mobile phase only. Raw sensitivity of RFRP3 7.7e5. Additionally shown retention of kisspeptin 10 and leuprolide.

extra washing the sample received on the trapping column, the matrix suppression was almost totally removed. This gave a rise in RFRP3 signal strength relative to the direct injection. With this chromatography system we also had good retention for leuprolide (used as an IS) and for kisspeptin 10 (other peptides of interest) as required for future assays (FIGURE 8).

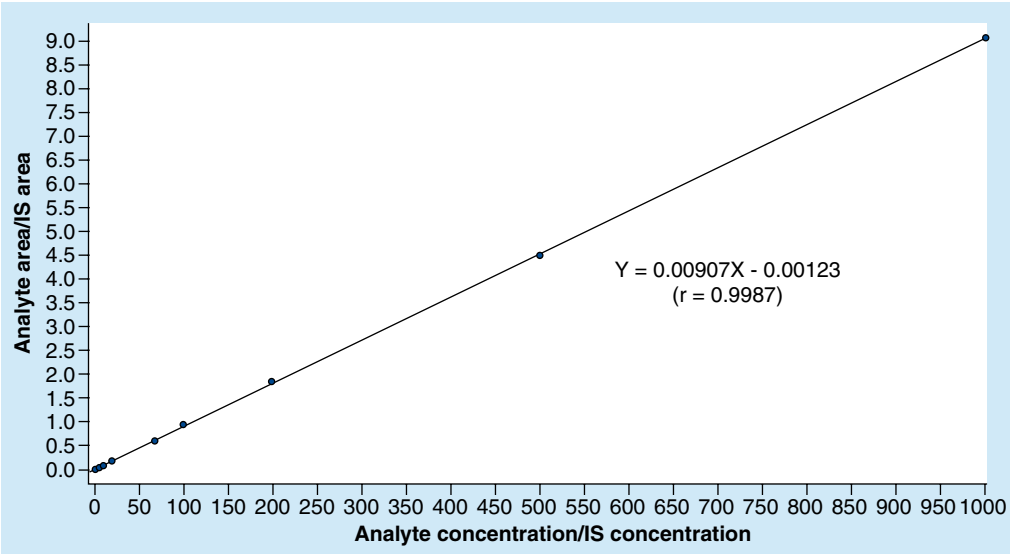

Figure 9. Calibration graph for day 0 acetonitrile crashed samples. Linear $1/X^2$ regression. IS: Internal standard.

Method validation

The first validation test batch using acetonitrile showed good linearity (r^2 = 0.9987) with good precision (mean CV across QC samples 3.9%) and accuracy (mean Accuracy across QC samples 105.5%; Table 4 & Figure 9) The tests for matrix

Table 4. Linearity and overnight stability of RFRP3 crashed with acetonitrile.

	Peak area (counts)	Concentration (ng/ml)	Accuracy (%)
Sample day 0			
Double blank	17		
Single blank	17		
Std 0.5 ng/ml	1330	0.501	100
Std 1 ng/ml	2210	1.01	101
Std 2 ng/ml	3480	1.97	98.3
Std 5 ng/ml	8860	5.09	102
Std 10 ng/ml	18,600	10.3	103
Std 20 ng/ml	32,300	17.7	88.5
Std 50 ng/ml	90,100	50.1	100
Std 100 ng/ml	190,000	105	105
Std 200 ng/ml	356,000	205	102
Std 500 ng/ml	905,000	497	99.4
Std 1000 ng/ml	1,760,000	1000	100
Single blank	663		
Single blank	41		
QC 5 ng/ml	9690	5.21	104
QC 5 ng/ml	9260	5.28	106
QC 50 ng/ml	99,400	50.8	102
QC 50 ng/ml	96,700	50.3	101
QC 500 ng/ml	1,030,000	560	112
QC 500 ng/ml	1,020,000	539	108
Sample day 1			
Double blank	32.6		
Single blank	20.6		
Std 0.5 ng/ml	1460	0.493	98.6
Std 1 ng/ml	2100	1.11	111
Std 2 ng/ml	3010	1.75	87.5
Std 5 ng/ml	8020	4.9	98.1
Std 10 ng/ml	13,600	8.66	86.6
Std 20 ng/ml	33,100	20.9	105
Std 50 ng/ml	74,100	48.8	97.5
Std 100 ng/ml	175,000	106	106
Std 200 ng/ml	333,000	218	109
Std 500 ng/ml	820,000	514	103
Std 1000 ng/ml	1,480,000	981	98.1
Single blank	544		
Single blank	101		
QC 5 ng/ml	8200	5.2	104
QC 50 ng/ml	77,400	50.3	100.6
QC 500 ng/ml	811,000	503	100.6
Single blank	566		
QC 50 ng/ml stored 20 h at -20°C	38,900	29.1	58.3
QC 50 ng/ml stored 20 h at -20°C	52,600	29.9	59.9
QC 50 ng/ml stored 20 h at RT	1790	1.18	2.36
QC 50 ng/ml stored 20 h at RT	2100	2.76	5.53

RT: Room temperature.

effect and recovery gave a mean recovery of 84.3% and matrix suppression of 12.9% (Table 5). However, when reviewing the longer term stability in the 'day 1' batch (Table 4) (samples stored in acetonitrile precipitate over night), there was a very noted degradation of compound. Samples stored at -20°C showed a 50% reduction in RFRP3 and samples stored at RT showed almost 95% loss of RFRP3. When this experiment was repeated, using methanol in place of acetonitrile as the denaturing solvent, again the first batch ('day 0') showed good linearity, precision (mean CV across QC samples 5.9%) and accuracy (mean accuracy across QC samples 107.5%). However, with a methanol crash, good stability for samples stored over night at both RT and -20°C was demonstrated (Table 6). Subsequently, this longer term storage was repeated after 5 days and again accuracy on QC samples remained acceptable, (within ±20% nominal [Data not shown]). Methanol was chosen as the denaturing solvent of choice for all *in vivo* assays.

The only notable difference recorded between the samples precipitated with acetonitrile and those precipitated with methanol was that the samples precipitated with methanol gave a fine powdery precipitate when the methanol was added, while the sample precipitated with acetonitrile gave much larger solid clumps of precipitate. This more powdery precipitate may lead to improved efficiency for inhibiting enzymatic activity.

In vivo sample data

The calibration data for the initial *in vivo* infusion sample set again was well within our normal discovery acceptance criteria (±20 accuracy and precision). Figure 10 shows chromatograms for RFRP3 at 0.2 ng/ml and a blank blood sample.

The data acquired most clearly showed the rapid turnover of RFRP3 to the hexamer and to some degree the pentamer (Figure 11).

Table 5. Recovery and matrix effects of RFRP3 crashed with acetonitrile.

	QC @50 ng/ml	Overspiked blank	Neat solution
	90,100	112,000	128,000
	99,500	113,000	131,000
	96,800	114,000	130,000
Mean	**95,500**	**113,000**	**130,000**
% Recovery	84.5		
% Matrix supression		13.1	

Conclusion

Endogenous signalling peptides prove to be very difficult to fully stabilize in very aggressive biological matrices such as whole rat blood. Some success has previously been reported for stabilization in human plasma, however, when monitoring attempts at a similar stabilization in whole blood from animal species, little to no success was

Table 6. Linearity and overnight stability of RFRP3 crashed with methanol.

	Peak area (counts)	Concentration (ng/ml)	Accuracy (%)	Precision (%)
Sample Day 0				
Double blank	0			
Single blank	0			
Std 5	10,500	4.98	99.6	
Std 10	20,100	9.76	97.6	
Std 20	39,900	21.5	107	
Std 50	92,600	48.4	96.8	
Std 100	201,000	102	102	
Std 200	404,000	199	99.4	
Std 500	1020,000	533	107	
Std 1000	1610,000	906	90.6	
Single blank	489			
Single blank	159			
QC100	218,000	116	116	
QC100	216,000	110	110	
QC100	210,000	111	111	2.9
Sample Day 1				
Double blank	0			
Single blank	0			
Std 5	8560	4.97	99.6	
Std 10	16,400	9.97	97.6	
Std 20	32,800	19.9	107	
Std 50	88,300	52.4	96.8	
Std 100	189,000	107	102	
Std 200	353,000	203	99.4	
Std 500	858,000	487	107	
Std 1000	1570,000	910	90.6	
Single blank	489			
Single blank	159			
QC100	176,000	109	109	
QC100	165,000	97.8	97.8	
QC100	173,000	94.2	94.2	7.7
QC100 stored 20 h at -20°C	208,000	120	120	
QC100 stored 20 h at-20°C	201,000	115	115	
QC100 stored 20 h at -20°C	208,000	120	120	2.4
QC100 stored 20 h at RT	209,000	114	114	
QC100 stored 20 h at RT	203,000	116	116	
QC100 stored 20 h at RT	206,000	122	122	3.5

RT: Room temperature.

observed. Direct sampling of whole blood into methanol stabilizes these peptides at their concentrations at the time of sampling. This method of stabilization, however, brings its own challenges around sample volume and cleanup limitations. In addition, the chromatography to simultaneously monitor for both the larger whole peptide and smaller cleaved fragments can be challenging owing to vastly different physicochemical behavior. These problems have been circumvented by the use of: selective *m/z* transitions to minimize cross talk, and the use of semi-2D chromatography to both preconcentrate (allowing much larger injection volumes) and prewash the samples before chromatographic separation (in lieu of cleaner sample preparation techniques). This can all be performed by the use of small sub-2-µm columns to perform full sample assay inside 3 min whilst maintaining selectivity and specificity. Being able to run standard LC with normal flow rates, rather than heading into nanoflow systems more usually associated with peptide analysis[12], gives a robust system that can be easily operated within an open-access environment and gives rapid sample turnarounds within the rapid timelines required in discovery bioanalysis. By enabling the use of large injection volumes (250 µl) , prefocused, onto overrun, narrow bore, Sub-2-µm columns, very high sensitivity levels can be generated due to the narrow final peak widths repeated. By simultaneously monitoring for cleaved fragments, which appear in higher concentrations than the parent they are derived from, it is possible to monitor for markers of peptide presence when even the very

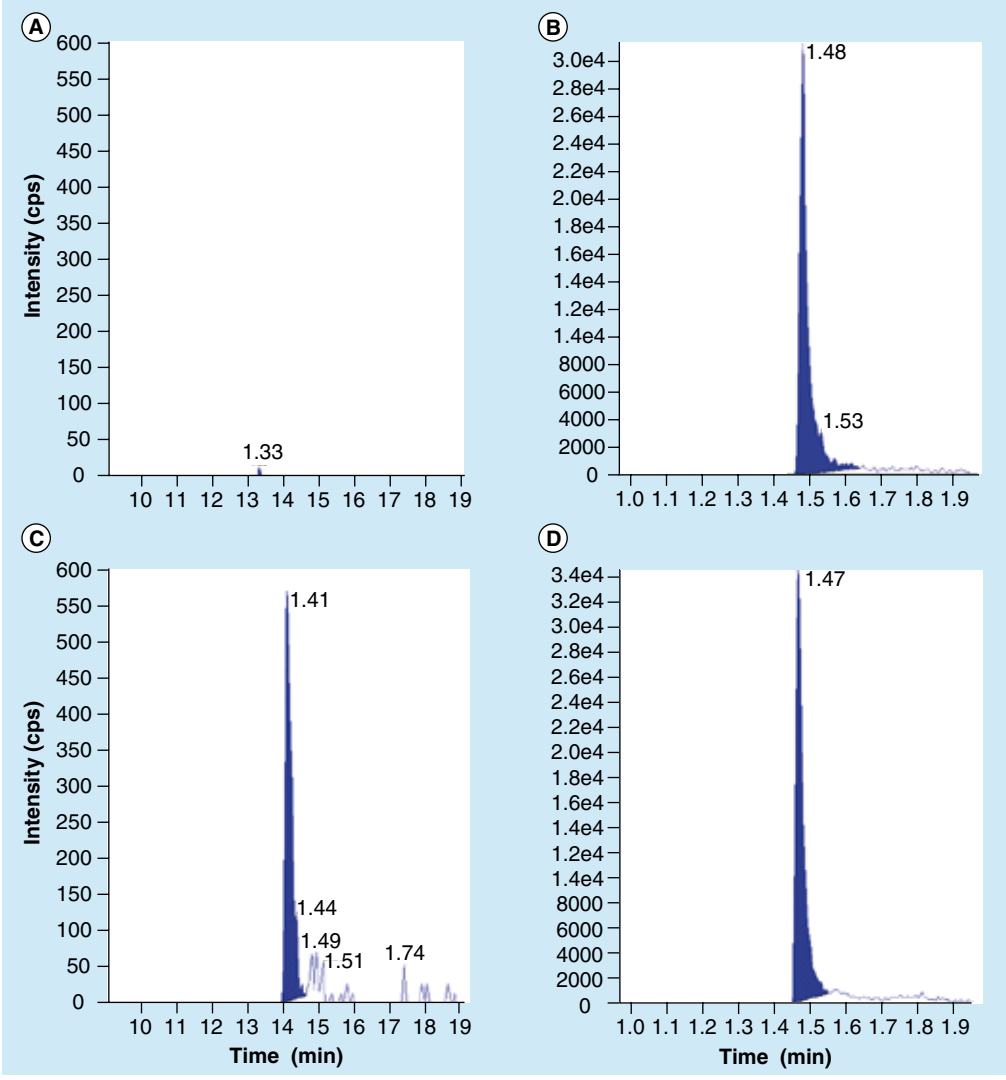

Figure 10. Chromatograms of RFRP3 samples and IS. (A) RFRP3 from blank with IS, **(B)** IS from blank with IS, **(C)** RFRP3 from LLOQ and **(D)** IS from LLOQ.
IS: Internal standard.

high sensitivity levels obtained remain insufficient to detect the presence of the parent peptide. By using this system, further assays have been set up and run for kisspeptin 10 (and fragments) and leuprolide showing its usability as a generic system for the assay of small signalling peptides.

Future perspective

Further sensitivity can be gained on theses assays by transferring them to next-generation MS platforms (i.e., API 5500 in place of the API 4000 used here). Sensitivity gains in the region of 10–50-fold have preliminarily been seen in house when making this transition [NOT SHOWN]. The linearity of loading on this LC system allows for very large injection volumes to be preconcentrated on to narrow-bore chromatography systems enabling fully scalable assays to larger sample volume where species allows.

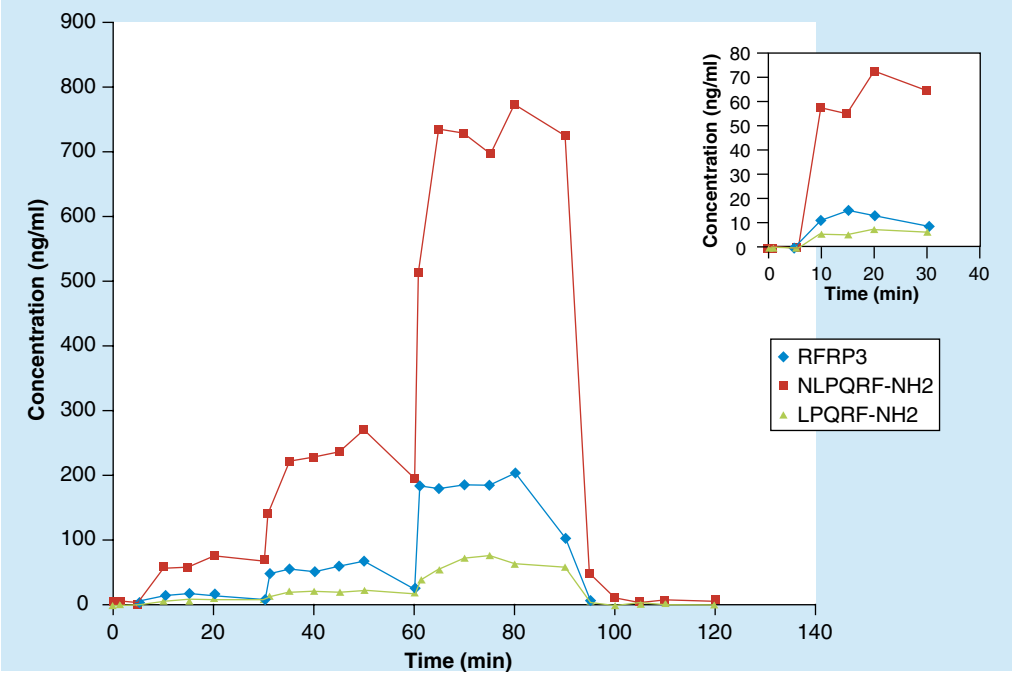

Figure 11. RFRP3, hexamer and pentemer concentration levels from *in vivo* samples infused with 500 µl/1800 /kg for 30 min then 1670 µl/1800 /kg for 30 min then 5000 µl/1800 /kg for 30 min of a 0.6 mg/ml solution of RFRP3.

Executive summary

- The small signaling peptide RFRP3 (VPNLPQRF-NH2) is hugely unstable in whole rat blood, rapidly suffering N-terminus cleavage down to the hexamer and pentamer. Stabilization of this peptide through the use of protease inhibitors proves extremely difficult (verging on impossible) in whole blood from species with high enzyme activity such as rats.
- Some success can be achieved with stabilization in plasma by the use of extremely high concentrations of protease inhibitors (e.g., aprotinin). However, even this has little effect on the stability in whole rat blood.
- With the extremely short half-life in whole blood (~ 6–8 min) even the time taken to generate plasma from blood would give hugely inaccurate readings for the peptide levels at time of sampling. As such, direct sampling into methanol is recommended to instantly deactivate all enzymatic activity.
- Direct sampling into solvent, although solving stability issues, raises challenges round sample preparation and detection due to receiving samples prediluted in large volumes of solvent. By use of 2D-LC, precipitated samples can be prewashed and preconcentrated onto a trapping cartridge. This allows for direct injection of large volumes of 'dirty' supernatant, that gets concentrated and cleaned, before elution into a narrow bore LC system.
- Through careful choice of *m/z* transitions and mobile phase composition, both parent peptide and the N-terminus cleaved fragments generated in blood can all be simultaneously monitored.
- Extremely sensitive assays (100 pM from 25µl samples) can be performed using standard flow LC with sub-2-µm particle columns enabling easy use open-access system for the assay of small peptides with straight-forward sample preparation and rapid run times (3 min).

Ethical conduct of research

The authors state that they have obtained appropriate institutional review board approval or have followed the principles outlined in the Declaration of Helsinki for all human or animal experimental investigations. In addition, for investigations involving human subjects, informed consent has been obtained from the participants involved.

Financial & competing interests disclosure

The authors have no relevant affiliations or financial involvement with any organization or entity with a financial interest in or financial conflict with the subject matter or materials discussed in the manuscript. This includes employment, consultancies, honoraria, stock ownership or options, expert testimony, grants or patents received or pending, or royalties.

No writing assistance was utilized in the production of this manuscript.

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- **Good wide-ranging review of the current state of peptide analysis by LC–MS/MS and the challenges presented by this analysis.**