Ion mobility separation of recombinant and synthetic insulin variants on the Cyclic IMS mass spectrometer

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Please reach out to me if you have any questions or comments – I'd love to discuss our work with you.
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Introduction

According to the World Anti-Doping Agency, synthetic insulins are prohibited substances in professional sport. Due to their peptidic character and largely identical amino acid sequences, their physico-chemical properties are very similar. Fig. 1 shows the amino acid sequence of human insulin and three analogs (lispro, glulisine and aspart). Human insulin and lispro insulin differ in the amino acid sequence at position B28/29 only and exhibit identical molecular masses. Separation of these analogs thus represents a considerable challenge, both chromatographically and by mass spectrometry (MS). Nevertheless, confident identification of these target peptides is crucial in doping controls as well as in forensics or related disciplines. Here, we evaluated cyclic ion mobility (IM) mass spectrometry to separate synthetic insulins, and its value for anti-doping analyses.

Experimental

All experiments were conducted on a Waters SELECT SERIES™ Cyclic IMS QToF (Manchester, UK). Static infusion experiments were conducted with pure insulin therapeutics using gold-coated glass capillaries. Standard flow LCMS of insulin used a Waters ACQUITY™ UPLC I-Class Plus with ACQUITY UPLC CSH C18 Peptide Column (130Å, 2.1mm*50mm, 1.7µm, #186006936). The gradient was 15-35% B over 9 min (0.2 ml/min, mobile phase A: water, formic acid 0.1%, DMSO 1%, B: acetonitrile, DMSO 1%). Finally, as a mimic of anti-doping samples, the insulin analogs were spiked into extracted serum at 5 ng/ml, and then immunoaffinity purified into 2% aqueous acetic acid. They were analysed on an ACQUITY™ UPLC M-Class and IonKey™ at 2µl/min (15 min run time with 15-40% B over 7 min; A: 0.1% formic acid; B: 0.1% formic acid in acetonitrile).

IM separation was optimised on the +5 charge state of the precursor ions by increasing the separation time and thus number of passes of the ions within the device (see Fig. 2). High energy fragmentation scans were also collected throughout the acquisitions (HDMSe method).

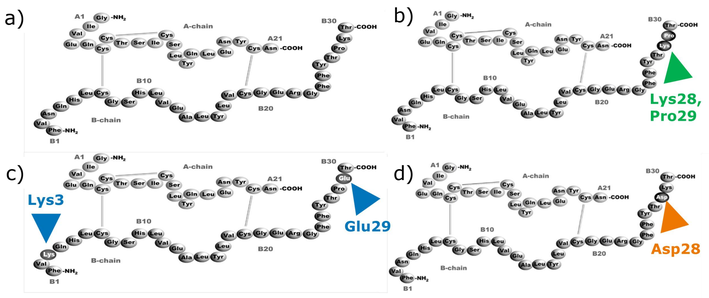

Fig 1: Sequences of a) human insulin, b) insulin lispro, c) insulin glulisine and d) insulin aspart

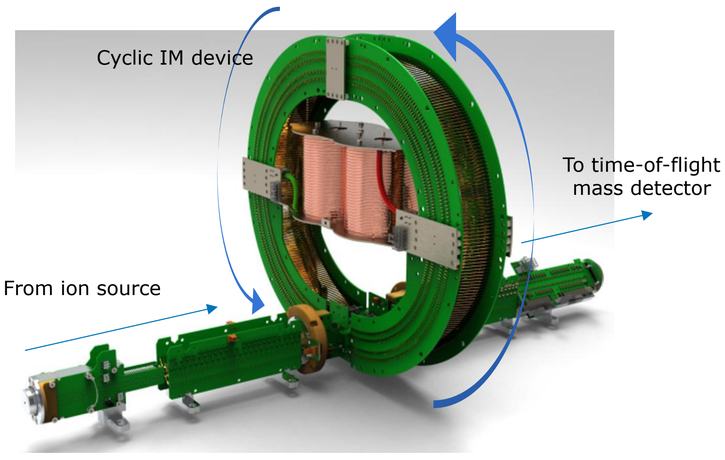

Fig 2: Model of the cyclic ion mobility device showing the path of ions (blue arrows). As ions pass through the mass spectrometer, they are accelerated in a perpendicular direction around an IM 'racetrack'. Increasing the number of passes around this racetrack leads to greater separation of ions with different collisional cross-sections.

Results

Using cyclic ion mobility coupled to high resolution MS, all four insulin variants were separated without chromatography or tandem MS. Fig. 3 shows the separation of human insulin and insulin lispro with direct infusion (i.e. without chromatographic separation before the Cyclic IMS mass spectrometer). Recombinant human insulin and insulin lispro are identical in the mass of their precursor ions (Fig 3a), but can be separated by IM (Fig 3b). Insulin lispro travels through the IM device faster than recHum insulin, resulting in a shorter drift time. The insulin analogs could be separated with 5-9 passes around the IM device (5 passes were chosen for the LCMS experiments).

Next, liquid chromatography (LC) was introduced before the mass spectrometer (Fig 4). LC could not separate a mixture of recHum and lispro insulin (Fig 4a, top panel). However, subsequent IM separation could separate them (Fig 4b, top panel). Thus, including IM within the LCMS acquisition solves the challenge of distinguishing these two isobaric insulin analogs.

Finally, the synthetic insulins in extracted serum were analysed, to test whether the method could be used to distinguish doping agents within the complex matrices handled in an anti-doping lab. The samples were immunoaffinity purified and analysed at low flow to give the greatest sensitivity. In a mixture of all four analogs, each of the synthetic insulins could be distinguished (Fig 4a), either on mass (recHum vs glulisine), drift time (recHum vs lispro) or both (recHum vs aspart). This identification is only possible when LC, IM and MS are all combined, as the individual techniques could not separate the mixture of analogs (Fig 4b). We had previously demonstrated the utility of LC-IM-MS for identification of insulins (Ref 1). However, the high mobility resolution achievable with the Cyclic IMS mass spectrometer allows confident identification of all four analogs within a mixture, even the isobaric human and lispro insulins. It thus facilitates the detection of illicit use of insulin lispro in sport.

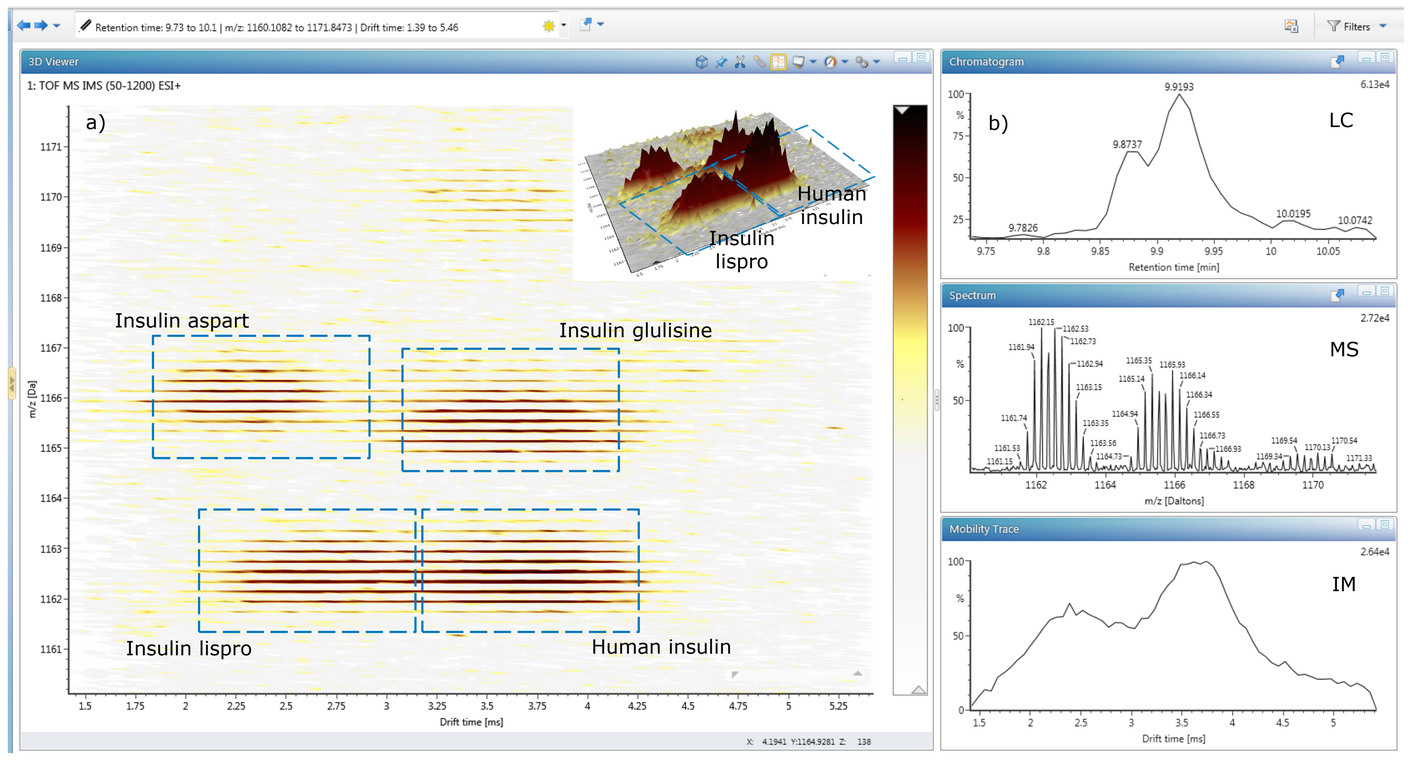

Fig 5: A mixture of 1 recHum: 1 lispro: 1 glulisine: 1 aspart in extracted serum (1.25 ng/ml each) can be separated by IM and MS. a) The m/z vs IM drift time plot within UNIFI™ software shows each analog has a different combination of m/z and drift time. The separation of recHum and lispro is clearer when the plot is viewed in 3D (insert, z-axis is intensity of ions). LC, MS and IM are not individually sufficient to separate the mixture (b).

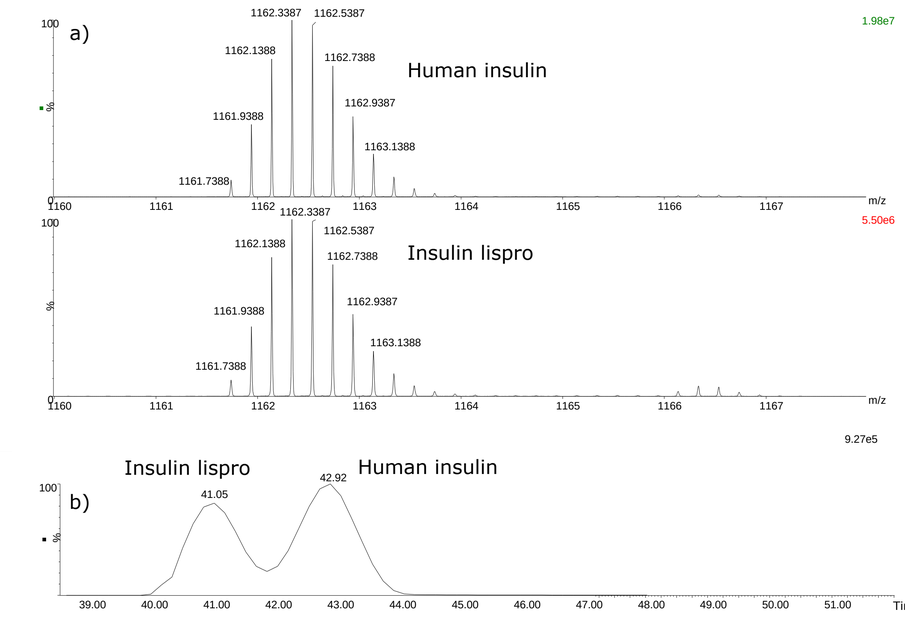

Fig 3: Human insulin and lispro insulin are indistinguishable by mass spectrum (a), but can be separated by ion mobility (b). IM mobilogram shows abundance of ions detected (y-axis) with different drift times through the cyclic IM device (x-axis), for the +5 charge state of a 1:1 mixture of recHum and lispro insulin (2.1 µM of each in 33% MeOH). Separation time = 28.55 ms (9 passes).

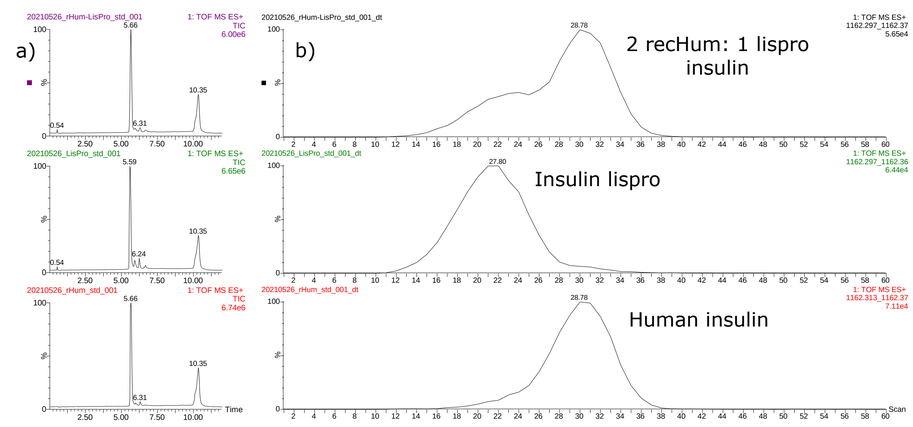

Fig 4: Liquid chromatography does not separate a 2:1 mixture of recHum and lispro insulin (a); but IM does (b). Ion mobilogram of +5 charge state shown. Separation time = 15.20 ms (5 passes).

Conclusion & outlook

- IM separation capability is significantly enhanced with the SELECT SERIES Cyclic IMS.
- The Cyclic IMS mass spectrometer was able to separate three synthetic forms (aspart, glulisine and lispro) of insulin from recombinant human insulin by IM.
- For insulin aspart and glulisine, the drift time separation complements the *m/z*-based separation, and will enhance confidence in the identification of these variants in unknown samples.
- For insulin lispro, its shorter drift time profile distinguishes the synthetic drug from isobaric human insulin. To be identified, these peptides have previously required retention time separation with long gradients and MS/MS experiments. Cyclic ion mobility therefore improves efficiency and confidence in their detection.
- IM separation can be combined with LC, MS and high energy MS scans (LC-HDMSe). Thus, the analyst can acquire drift time, m/z of the precursor, m/z of peptide fragments and retention time within a short experiment.
- Detection of mixtures of insulins was demonstrated within complex matrices typical of anti-doping studies. Further work will establish the limit of detection of this method to distinguish insulin lispro from human insulin (goal is ≤ 0.5 ng/ml for anti-doping applications).
- Combining existing sample preparation and chromatographic strategies with novel cyclic IM HRMS capabilities can substantially improve tests for synthetic insulins in sports and forensics.

References

[1] Thomas A, Schänzer W, Thevis M., Determination of human insulin and its analogues in human blood using liquid chromatography coupled to IM mass spectrometry (LC-IM-MS). Drug Test Anal. 2014 Nov-Dec;6(11-12):1125-32. doi: 10.1002/dta.1710.

Acknowledgments

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