The measurement of KRAS G12 mutants using multiplexed selected reaction monitoring and ion mobility mass spectrometry

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Rationale: There is a considerable clinical demand to determine key mutations in genes involved with cancer which necessitates the deployment of highly specific and robust analytical methods. Multiplex liquid chromatography with selected reaction monitoring (LC/SRM) assays offer the ability to achieve quantitation down to levels expected to be present in clinical samples. Ion mobility mass spectrometry (IMS/MS) assays can provide increased peak capacity and hence separation in an extremely short time frame, and in addition provide physicochemical data regarding the collision cross-section of an analyte which can be used in conjunction with the m/z value of an ion to increase detection specificity.

Methods: For LC/SRM, unlabelled peptides and corresponding stable-isotope-labelled standards were spiked into digested human plasma and analysed using ultrahigh-performance liquid chromatography (UHPLC) coupled to a triple quadrupole mass spectrometer to enable the generation of analyte-specific calibration lines. Synthetic unlabelled peptides were infused into a Synapt G2 mass spectrometer for travelling wave ion mobility separation and TWCCS values were derived from comparison with previously generated TWCCS calibration values.

Results: Linear calibration lines (0.125 to 25 fmol/μL) were established for each of the KRAS peptides. UHPLC separated the peptides and hence enabled them to be split into different retention time functions/windows. This separation enabled detection of three or four transitions for each light and heavy peptide with at least 10 points per peak for accurate quantitation. All six KRAS G12 peptides were separated using IMS/MS, enabling precise TWCCS values to be determined. Although some of the G12 peptides chromatographically co-eluted, all the peptides were distinguished by m/z, retention time and/or drift time.

Conclusions: This study advocates that LC/SRM and IMS/MS could both be used to identify single amino acid substitutions in KRAS as an alternative to commonly used methods such as circulating tumour DNA analysis.
1 | INTRODUCTION

Targeted liquid chromatography with selected reaction monitoring (LC/SRM) assays have great potential for use in the clinic to detect protein biomarkers for the diagnosis and monitoring of a wide variety of diseases. Despite offering many advantages, significant challenges still exist and a very limited number of LC/SRM-based assays have been adopted in the clinical setting to date. Implementing this technique in clinical laboratories necessitates the development of robust, quantitative assays for clinically relevant biomarkers for which reliable detection methods do not currently exist. In addition, if the assay is unable to provide a financial operation cost advantage, the information derived from the assay has to be of superior quality to that provided by other methods.

KRAS is a protein first identified in the Kirsten rat sarcoma virus and is involved in cell signalling which activates cell growth in response to growth factor binding. It contains an intrinsic GTPase which switches off the pathway; however, single amino acid substitutions can inactivate the GTPase. This leads to constitutive activation of KRAS and aberrant growth. KRAS is implicated in many cancers including colorectal cancer, pancreatic cancer and non-small cell lung cancer. Amino acid substitutions are driver mutations, cancers including colorectal cancer, pancreatic cancer and non-small cell lung cancer. KRAS gene with substitutions of the glycine at position 12 is the most common. KRAS has an extremely low copy number and has not previously been detected in human plasma. Consequently, determination of KRAS status is primarily undertaken using genetic testing. However, genetic testing relies on invasive, costly and time-consuming (typically around 7 days) methodologies which rely on high-fidelity amplification to achieve genetic determination.

Travelling wave ion mobility separation (TWIMS) enables gas-phase electrophoretic separation by creating a dynamic pulse of ions within a dense gas-filled chamber (in this case nitrogen). Ions are separated by their size, shape or charge state. Since mutated peptides differ by a single amino acid and some of them co-elute using reversed-phase LC/SRM, we also investigated the use of TWIMS to improve separation of peptides and determine the collision cross-section (CCS) values, thus potentially allowing the use of this parameter to specifically identify the mutations in biological samples. Ion mobility separates ions in milliseconds, nesting between ultra high-performance liquid chromatography (UHPLC) which separates in seconds and time of flight which separates on a timescale of microseconds, resulting in increased peak capacity and offering the ability to analyse complex samples in a very short amount of time which could be advantageous in the clinical setting. Peptides unique to each RAS protein were selected to distinguish between the RAS isoforms, NRAS, HRAS and KRAS, because the tryptic peptide containing the mutation is found in all isoforms. Stable-isotope-labelled (heavy) standards were incorporated into the LC/SRM assay to enable absolute quantification of the peptides using isotope dilution and multipoint calibration lines.

2 | MATERIALS AND METHODS

2.1 | Materials

All chemicals and reagents were purchased from Sigma Aldrich (Poole, UK) or Fisher Scientific (Loughborough, UK) unless otherwise stated. Stable-isotope-labelled (heavy) peptides were purchased from Pepscan (Lelystad, The Netherlands) and unlabelled (light) custom-synthesised peptides were acquired from Genecust (Ellange, Luxembourg).

2.2 | Liquid chromatography

For LC/SRM analysis, samples were injected onto a NanoAcquity UHPLC system with a 2G-V/M Symmetry C18 trap column (180 μm × 20 mm, 5 μm) for desalting and chromatographic focusing before elution onto an Acquity HSS T3 analytical UHPLC column (75 μm × 250 mm, 1.8 μm) (all from Waters, Milford, MA, USA). The analytical column temperature was set at 40°C and the auto sampler temperature was maintained at 6°C. Trapping occurred for 3 min with 99.9% solvent A and 0.1% solvent B at a flow rate of 5 μL/min. A 60 min liquid chromatography gradient was initiated on elution from the trap column. The following gradient was used: 0 min, 3% B; 40 min, 50% B; 40.33 min, 85% B; 51.60 min, 85% B; and 52 min, 3% B. The flow rate was set at 0.3 μL/min. Solvent A was LC/MS-grade water containing 0.1% formic acid. Solvent B was acetonitrile containing 0.1% formic acid.

2.3 | Mass spectrometry

A Waters Xevo TQ mass spectrometer was operated in positive electrospray ionization mode. The capillary voltage was set at 2.40 kV and the cone voltage was set at 30 V. Argon was used as the collision gas and the collision energy was optimized for each peptide by repeated injections using different collision voltages. Initially the precursor ion for each peptide was determined by performing a mass spectrometry (MS) scan followed by a product ion scan to identify the most abundant product ions to select as the SRM transitions. Exact m/z values were extracted from the raw data using Skyline software (MacCoss Laboratory). Tryptically digested peptides at residues 89–97 are unique to each RAS protein and were used to distinguish between KRAS, HRAS and NRAS. The RAS-specific peptide sequences were SFEDIHQYR, SFEDIHYYR and SFADINLYR for HRAS, KRAS and NRAS, respectively. The RAS G12 tryptically digested peptide spanned residues 6–16 (LWVGGGAGGG). SRM transitions and optimized collision energies for each peptide are presented in Table 1. Human volunteer plasma (25 μL) was added to
275 μL of 50 mM ammonium bicarbonate, pH 7.8. Dithiothreitol was added to a final concentration of 15 mM and incubated for 30 min at 60°C. Iodoacetamide was added to a final concentration of 20 mM and incubated for 30 min in the dark at room temperature. Then, 8 μL of 5 μg/μL trypsin (dissolved in ammonium bicarbonate, pH 7.8) was added and the sample was left overnight at 37°C. On the next day, 3 μL of formic acid was added to stop trypsinolysis. The sample was freeze-dried overnight, then reconstituted in 1 mL of 0.1% formic acid and acetonitrile (97:3), and split into 100 μL aliquots and stored at −80°C. Calibration lines were generated by spiking into 20 μL of the digested plasma the nine peptide standards ranging from 0.125 to 25 fmol/μL and 25 fmol/μL of the equivalent heavy-labelled standard.

A 500 fmol/μL mixture of wild-type and five mutant KRAS 6–16 peptides was mixed in a ratio of 1:1 with the digested plasma solution described above and infused into a Waters Synapt G2 mass spectrometer at a flow rate of 1 μL/min. The instrument was operated in positive ion electrospray mode. The capillary voltage was set at 2.40 kV and the cone voltage to 35 V. The flow rate of the nitrogen in

| Peptide | Protein | Precursor m/z (light) | Product m/z (light) | Precursor m/z (heavy) | Product m/z (heavy) | Collision energy (eV) |
|---------|---------|-----------------------|---------------------|-----------------------|---------------------|-----------------------|
| SFEDIHQYR | HRAS | 398.8561 | 302.1535 | 402.1922 | 307.1577 | 15 |
| SFEDIHQYR | HRAS | 398.8561 | 358.6956 | 402.1922 | 363.6997 | 15 |
| SFEDIHQYR | HRAS | 398.8561 | 480.7303 | 402.1922 | 485.7345 | 15 |
| SFEDIHQYR | KRAS | 401.8562 | 363.1957 | 405.1923 | 368.1998 | 15 |
| SFEDIHQYR | KRAS | 401.8562 | 475.2412 | 405.1923 | 485.2495 | 15 |
| SFEDIHQYR | KRAS | 401.8562 | 558.7647 | 405.1923 | 563.7688 | 15 |
| LVVVGAGGVGK | Ras WT | 478.3004 | 372.2241 | 482.3075 | 376.2312 | 17 |
| LVVVGAGGVGK | Ras WT | 478.3004 | 545.3042 | 482.3075 | 553.3184 | 17 |
| LVVVGAGGVGK | Ras WT | 478.3004 | 644.3726 | 482.3075 | 652.3868 | 17 |
| LVVVGAGGVGK | Ras WT | 478.3004 | 743.4411 | 482.3075 | 751.4552 | 17 |
| LVVVGAGGVGK | Ras G12A | 485.3082 | 502.2984 | 489.3153 | 510.3126 | 18 |
| LVVGAAGVGK | Ras G12A | 485.3082 | 559.3198 | 489.3153 | 567.334 | 18 |
| LVVGAAGVGK | Ras G12A | 485.3082 | 659.3882 | 489.3153 | 666.4024 | 18 |
| LVVGAAGVGK | Ras G12A | 485.3082 | 757.4567 | 489.3153 | 765.4709 | 18 |
| LVVVGAGGVGK | Ras G12S | 493.3057 | 387.2294 | 497.3128 | 391.2365 | 17 |
| LVVVGAGGVGK | Ras G12S | 493.3057 | 575.3148 | 497.3128 | 583.3289 | 17 |
| LVVVGAGGVGK | Ras G12S | 493.3057 | 647.3832 | 497.3128 | 682.3974 | 17 |
| LVVVGAGGVGK | Ras G12S | 493.3057 | 773.4516 | 497.3128 | 781.4658 | 17 |
| LVVVGAVGVGK | Ras G12V | 499.3239 | 587.3511 | 503.331 | 595.3563 | 18 |
| LVVVGAVGVGK | Ras G12V | 499.3239 | 686.4196 | 503.331 | 694.4338 | 18 |
| LVVVGAVGVGK | Ras G12V | 499.3239 | 785.4888 | 503.331 | 793.5022 | 18 |
| LVVVGADGVGK | Ras G12D | 507.3031 | 546.2882 | 511.3102 | 554.3024 | 18 |
| LVVVGADGVGK | Ras G12D | 507.3031 | 603.3097 | 511.3102 | 611.3239 | 18 |
| LVVVGADGVGK | Ras G12D | 507.3031 | 702.3781 | 511.3102 | 710.3923 | 18 |
| LVVVGADGVGK | Ras G12D | 507.3031 | 801.4465 | 511.3102 | 809.4607 | 18 |
| LVVGAC(+57)GVGK | Ras G12C | 529.805 | 591.2919 | 533.8121 | 599.3061 | 19 |
| LVVGAC(+57)GVGK | Ras G12C | 529.805 | 648.3134 | 533.8121 | 656.3276 | 19 |
| LVVGAC(+57)GVGK | Ras G12C | 529.805 | 747.3818 | 533.8121 | 755.396 | 19 |
| LVVGAC(+57)GVGK | Ras G12C | 529.805 | 846.4502 | 533.8121 | 854.4644 | 19 |
| LVVGAC(+57)GVGK | Ras G12C | 529.805 | 945.519 | 533.8121 | 963.5338 | 19 |
| SFADINLYR | NRAS | 549.7282 | 421.1718 | 554.7867 | 421.1718 | 19 |
| SFADINLYR | NRAS | 549.7282 | 565.3093 | 554.7867 | 575.3175 | 19 |
| SFADINLYR | NRAS | 549.7282 | 793.4203 | 554.7867 | 803.4285 | 19 |
| SFADINLYR | NRAS | 549.7282 | 864.4574 | 554.7867 | 874.4657 | 19 |
the ion mobility separation (IMS) cell was 111 mL/min and the flow rate of helium in the helium cell was 180 mL/min. Different wave velocities were tested from 500 to 1400 m/s at 100 m/s intervals to obtain the optimum orthogonal separation. A wave velocity of 1330 m/s was ultimately chosen for maximal separation. For each sample, data were acquired for 5 min to improve the signal-to-noise ratio.

2.4 | CCS determination

A bovine serum albumin (BSA) digest standard (Waters Ltd, Elstree, UK) was analysed using a data-independent acquisition method which incorporates TWIMS which is known as high-definition MS$^E$ (HDMS$^E$). HDMS$^E$ allows the fragmentation of mobility-separated precursor ions. KRAS standard peptides were analysed under identical conditions to those for the BSA standards and the drift times (bins) determined for each peptide. Only [M + 2H]$^{2+}$ ions were selected for both calibrants and analyte ions. MS analyses were performed using HDMS$^E$ for LC/MS and IMS/MS with continuous infusion. The parameters for the MS analysis were as described above, with TWIMS wave height of 40 V and IMS wave velocity of 1330 m/s.

2.5 | Data analysis

TWIMS data were analysed using DriftScope software (Waters Corporation, Wilmslow, UK). LC/SRM data were analysed using Skyline. The unlabelled peptide retention times were confirmed by comparison with the heavy-labelled standards which contained $^{13}$C$_6$-$^{15}$N$_2$-lysine or $^{13}$C$_6$-$^{15}$N$_4$-arginine at the C-terminal, leading to a molecular mass increase of 8 or 10 Da, respectively. The unlabelled peptide peak areas were normalized to the heavy standard. The travelling wave CCS ($^{TW}$CCSN$_2$) was then calculated using a method described by Michaelevski et al.$^{15}$

The drift times of each of the peptides were obtained from the raw data following ProteinLynx Global Server (PLGS; Waters Corporation, Wilmslow, UK) processing of BSA peptides and KRAS mutant standards under the same MS conditions. The drift times of calibration ions and KRAS peptide ions were corrected to accommodate time spent outside the mobility region of the instrument using Equation (1):

$$t'_{0D} = tD - \frac{\sqrt{mz}}{1000}$$

where $t_D$ is the measured drift time of the precursor ion, $t'_{0D}$ is the corrected drift time of the precursor ion, $m/z$ is the mass-to-charge ratio of the precursor ion and $c$ is the enhanced duty cycle delay coefficient. A corrected DT was determined for BSA peptides and these times were plotted against known $\Omega'_{N2}$ values to establish the calibration line from the Bush Laboratory.$^{16}$ Values of $\Omega'_{N2}$ for KRAS mutant peptide standards were then derived using the coefficients from the calibration line. The corresponding $\Omega_{N2}$ values (absolute $^{TW}$CCSN$_2$) were obtained by factoring in the calculated reduced mass. IMS analysis for BSA and KRAS standards was ultimately performed using an IMS wave velocity of 1330 m/s.

3 | RESULTS AND DISCUSSION

A method was required to identify the various KRAS mutations which are commonly found in different tumours including pancreatic, colorectal and lung cancers. Using LC/SRM, the wild-type and five
most common mutant peptides were separated using a 60 min LC gradient and accurately quantified using stable-isotope-labelled standards; the RAS isoforms were also detected. A representative chromatogram and a calibration line constructed in digested human plasma are shown in Figure 1, demonstrating the linearity of quantification of the wild-type peptide. The separation of the RAS peptides using LC/SRM is shown in Figure 2. Four discrete retention time windows were used to enable the detection of 3–4 transitions for each peptide without compromising sensitivity. For each peptide the linear range was 0.125 to 25 fmol/μL, ensuring a sensitive assay for KRAS detection. Table 2 presents the lower limits of detection and quantitation for each of the peptides. A similar method has been developed by Wang et al14; however, this only detected the RAS-specific peptides and RAS wild-type, G12D and G12V peptides which are commonly found in pancreatic cancer. This LC/SRM method allows the potential for multiplex analysis of the whole RAS biology within biological samples.

In certain settings it may be appropriate to provide qualitative data which simply inform the clinician as to which mutation(s) are present. Therefore, analysis with IMS/MS using continuous infusion provides a potentially rapid capability of phenotyping patient samples. TWIMS allows highly specific identification of mutations in KRAS because the various mutant peptides can be separated by mobility in addition to the m/z value. Figure 3A shows the separation of the peptides spiked into digested human plasma in a three-dimensional ion mobility spectrum. Due to the infusion aspect of the IMS experiment, we can rapidly detect the different mutations of KRAS. Figure 3B shows the same analysis of digested human plasma without the peptides and it should be noted that the scale is 37.5 times greater in the KRAS peptide mobilogram. The MS spectra and mobilograms thus demonstrate that the ion mobility

| Peptide sequence | Retention time (min) | LLOD (fmol/μL) | LLOQ (fmol/μL) | R²   |
|------------------|---------------------|---------------|---------------|------|
| SFEDIHQYR        | 22.50               | 0.31          | 1.0           | 0.998|
| SFEDIHYR         | 21.30               | 0.50          | 1.0           | 0.996|
| LVVVGAGVYGK      | 23.95               | 0.21          | 1.0           | 0.999|
| LVVVGAGVYK       | 24.40               | 0.52          | 12.5          | 0.998|
| LVVVGASVYGK      | 23.87               | 0.24          | 1.0           | 0.998|
| LVVVGAGYGK       | 26.23               | 2.75          | 6.25          | 0.998|
| LVVVGAGDYGK      | 24.16               | 0.27          | 1.0           | 0.999|
| LVVVGAGCGYGK     | 24.23               | 0.31          | 1.0           | 0.998|
| SFADINLYR        | 28.37               | 0.24          | 6.25          | 0.998|

*LLOD and LLOQ are derived from Skyline using linear regression with ratio of heavy to light normalisation and for LLOD blank plus 2 × SD. Replicate coefficients of variation all below 5% for all concentrations except 0.125 fmol/μL which did not exceed 15% for any of the peptides.
provides sufficient specificity that is distinct from background signals. In addition to the \( m/z \) values, we can obtain TWCCSN2 values that are specific to the mutated peptides. Table 3 presents the TWCCSN2 values ultimately derived from the drift time measurement of the individual peptides. The TWCCSN2 values obtained for each peptide reflected \( m/z \) except for G12V which had a larger TWCCSN2 than G12D despite having a smaller \( m/z \) value. This is presumably because of the aliphatic nature of valine which might increase the TWCCSN2 of the peptide. The measurement of TWCCSN2 could be used to accurately determine the mutation status in a clinical or biological sample.

The TWCCS value is a constant and can be easily measured using TWIMS (the drift times were obtained as an average of three determinations with a coefficient of variation across the measurements of 0.14%). LC/MS is potentially susceptible to shift in the chromatographic retention times which, when looking for a single mutation, could potentially affect selectivity. These retention time shifts can of course be ameliorated by the use of standards. However, where there is chromatographic overlap in transition windows, even small retention time shifts may hinder the reliable measurement of peptides and thus the translation of the method into clinical laboratories. TWIMS offers considerably reduced analysis time.

**FIGURE 3** KRAS 6–16 peptide mix (wild-type and the five most common mutations) spiked (500 fmol/μL) into tryptically digested human plasma peptides analysed using IMS/MS via continuous infusion (1 μL/min). (A) MS spectrum obtained by extracting spectra from relevant region of IMS acquired data. Inset (left) shows mobility separated peptide ions whilst inset (right) is the same mobiogram but orientated approximately 90°. (B) Identical analysis of tryptically digested plasma in the absence of the KRAS 6–16 peptide mix. The extracted MS spectra show clear absence of interfering \( m/z \) peaks. Note: relevant region has been amplified 20-fold.

**TABLE 3** Detection of KRAS 6–16 peptide mix. Detection of individual KRAS peptides and the resultant TWCCSN2 estimations using a digested BSA peptide calibration line^a*

| KRAS variant | Sequence | MI mass | Measured \( m/z \) | Average DT bins | DT | DT' | Red. mass | \( \Omega' \) (Å²) | Calculated TW CCSN2 (Å²) |
|--------------|----------|---------|----------------|--------------|----|-----|----------|--------------|--------------------------|
| WT           | LVVVAGGVGK | 954.5862 | 478.2959 | 100.39 | 6.927 | 6.894 | 5.216 | 856.91 | 328.5 |
| G12A         | LVVVAGGVGK | 968.6019 | 485.3037 | 103.86 | 7.166 | 7.133 | 5.218 | 871.43 | 334.0 |
| G12S         | LVVVAGGVGK | 984.5968 | 493.3012 | 105.68 | 7.292 | 7.259 | 5.221 | 879.03 | 336.9 |
| G12V         | LVVVAGGVGK | 996.6332 | 499.3194 | 107.69 | 7.430 | 7.397 | 5.220 | 887.42 | 340.0 |
| G12D         | LVVVAGGVGK | 1012.592 | 507.2986 | 109.23 | 7.399 | 7.365 | 5.221 | 885.49 | 339.2 |
| G12C         | LVVVAGGVGK | 1057.595 | 529.8005 | 109.22 | 7.536 | 7.501 | 5.224 | 893.77 | 342.2 |

^aMI mass, monoisotopic mass; measured \( m/z \), acquired \( m/z \) of the peptide; average drift time bins \((n = 3)\); DT, drift time; DT', corrected drift time; red. mass, reduced mass; \( \Omega' \), normalized CCS value; TWCCSN2, CCS value derived in travelling wave ion mobility in nitrogen gas.
Instead of using the long chromatographic gradients required for sufficient liquid-phase separation, rapid profiling of samples could be achieved in several minutes using TWIMS, enabling a selective and specific method for the higher throughput analysis of samples. Sensitivity may be a disadvantage for TWIMS but the continual improvement in capture technologies and strategies will greatly enable clinical implementation of TWIMS and other IMS/MS approaches.

4 | CONCLUSIONS

The ability of TWIMS to differentiate the different KRAS mutant peptides demonstrates the distinct opportunity for this technology to provide a highly selective and specific method that can precisely discriminate KRAS mutants and thus allow the phenotyping of biological samples. It is demonstrated that, using previously generated $^{T_{w}}CC_{S2}$ calibration values, we could accurately determine the $^{T_{w}}CC_{S2}$ values of each of the KRAS mutant peptides for the first time. In addition, we have described a LC/SRM method that can allow quantitation of these clinically important KRAS mutations.

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