PAPER

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Development of a sensitive biochemical assay for the detection of tofacitinib adherence

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Rheumatoid arthritis (RA) is a chronic autoimmune inflammatory disease. Tofacitinib is a Janus Kinase inhibitor licensed for the treatment of RA that, unlike biologic anti-rheumatic drugs, is administered orally, but studies of long-term treatment adherence rates are lacking. The measurement of adherence, however, is challenging and there is currently no gold standard test for adherence. Here, we developed a novel HPLC MS/MS assay for the quantification of tofacitinib. The assay demonstrated a LLOQ for tofacitinib of 0.1 ng ml^{-1}, within run accuracy was 81–85% at LLOQ and 91–107% at all other levels. To investigate the ability of the assay to detect adherence, tofacitinib was measured in a random selection of serum samples ($n = 10$) of tofacitinib treated RA patients who self-reported adherent behaviour. The assay measured tofacitinib in all samples above the LLOQ demonstrating the potential of the assay to sensitively measure biochemical adherence in real-world patient samples. This method for detection of adherence has the potential to be a more objective measure that could be used in the future in the clinic but will require further studies to explore factors that may influence measurement of drug levels, such as clinical characteristics of patients.

Introduction

Tofacitinib is a Janus Kinase (JAK) inhibitor in tablet form that selectively inhibits the JAK1/3 tyrosine kinases available in 5 mg twice daily or once-daily 11 mg modified-release dosing. The JAK tyrosine kinases are intracellular signalling proteins, responsible for activating downstream Signal Transducer and Activator of Transcription (STAT) proteins that regulate gene transcription. JAK activation relies upon binding of cytokines to the type I and type II cytokine receptors, including proinflammatory cytokines such as type I interferons and IL-6.[1] Selective inhibition of the JAK tyrosine kinases is used to treat several chronic inflammatory diseases. In the United Kingdom (UK), tofacitinib is licensed for the treatment of rheumatoid arthritis (RA), psoriatic arthritis and ulcerative colitis.

In RA, a chronic inflammatory arthritis, early effective treatment has been shown to reduce joint erosions and disability.[2] Patients in the UK with severe, active RA that has failed to respond to conventional anti-rheumatic drug treatments are eligible for the second-line more effective biologic (b) or targeted synthetic (ts) disease modifying anti-rheumatic drugs (DMARDs), such as tofacitinib. Second-line therapy response is not, however, universal and up to 27% of RA patients do not take their subcutaneous bDMARD as prescribed resulting in significantly reduced response.[3] One of the most common side effects of subcutaneously administered bDMARDs is injection site reactions and injection site pain is a significant predictor of non-adherence.[4] Oral administration offers a major potential benefit to patients, removing the risk of injection site reactions. Frequency and route of administration may affect patients' adherence and patients prefer an oral DMARD.[5] Oral tofacitinib has been shown to be at least as effective as the traditional subcutaneous bDMARD for treating RA[6–8] but it is unclear whether adherence is higher.

Previous research utilising prescription claims has revealed no difference between adherence to tofacitinib and subcutaneous bDMARDs.[9–11] However, the use of indirect measures such as pharmacy refill data can be inaccurate indicating a need for a more accurate and objective adherence measurement. Tofacitinib levels *in vivo* can be measured using High Performance Liquid Chromatography Selected Reaction Monitoring Mass

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Spectrometry (HPLC-SRM-MS). The pharmacokinetic profile of tofacitinib using a simulated physiologically-based pharmacokinetic (PBPK) model demonstrates that a lower limit of quantification (LLOQ) of 0.1 ng ml^{-1} is sufficient for the detection of adherence following 5 mg twice daily administration.[12]

Without an objective direct measure of adherence, it remains uncertain whether the patient benefits of oral over subcutaneous DMARDs is associated with improved adherence. The aim of the present study is to, therefore, develop a sensitive novel HPLC-SRM-MS tofacitinib assay using the EMA guidelines as a benchmark[13] with exploration of the ability of the assay to detect adherence in patient samples.

Experimental

Materials and methods

Reagents and chemicals. Tofacitinib citrate and deuterium-labelled tofacitinib (tofacitinib-d3) were purchased from Cayman Chemical (Michigan, USA). LC-MS grade acetonitrile (ACN), water, pooled human serum and formic acid were purchased from Merck (Dorset, United Kingdom).

Preparation of standards and samples. Human serum for method development was obtained from healthy volunteers recruited to the collection of blood and urine samples from volunteers for the development of analytical methods study (UREC 12346) and the National Repository Study (REC 99/8/84) following informed consent.

Stock solutions of tofacitinib and tofacitinib-d3 were prepared in water at 100 μg ml^{-1} for tofacitinib and 500 μg ml^{-1} for tofacitinib-d3 and stored at −80 °C. Working solutions were prepared freshly for each batch of samples. Samples were prepared in 2 ml safe-lock tubes (Eppendorf®). Samples were spiked with tofacitinib and tofacitinib-d3 to give a final concentration of 50 ng ml^{-1} tofacitinib-d3 and final concentrations of tofacitinib at 0.1 ng ml^{-1}, 0.25 ng ml^{-1}, 0.5 ng ml^{-1}, 1 ng ml^{-1}, 2.5 ng ml^{-1}, 5 ng ml^{-1}, 10 ng ml^{-1}, 50 ng ml^{-1} and 100 ng ml^{-1}. Protein precipitation was performed by the addition of 200 μl ACN to 50 μl sample, samples were vortex mixed and then centrifuged at 10 000g for 10 minutes. The supernatant was removed and dried in a concentrator (Eppendorf concentrator plus) for 3 hours at room temperature and reconstituted in 50 μl water directly prior to LC-MS/MS analysis.

Chromatographic conditions and mass-spectrometry detection. LC-MS/MS analysis was performed on a TSQ Vantage triple quadrupole mass spectrometer coupled with an Accela UHPLC system (Thermo Fisher Scientific, Massachusetts, USA). Chromatographic separation was performed using a Thermo Scientific™ Hypersil GOLD™ HPLC column with a particle size 1.9 μm, 100 mm length and 2.1 mm diameter maintained at 25 °C. 10 μl of sample was injected onto the column. The mobile phase consisted of ACN with 0.1% formic acid as the organic component (B) and water with 0.1% formic acid as the aqueous phase (A). The system was maintained in 8% buffer B at a flow rate of 0.3 ml min^{-1}. Samples were maintained at 4 °C prior to analysis.

Tofacitinib and tofacitinib-d3 were detected using heated electrospray ionisation in positive ion mode using the following selected reaction monitoring (SRM) transitions: 313.0 > 173.06 for tofacitinib and 316.3 > 176.1 for tofacitinib-d3. The mass spectrometer settings were optimised as follows: spray voltage 5000 V, capillary temperature 369 °C, vaporiser temperature 353 °C and collision energy 29 eV. Argon was used as collision gas. Quantitation was calculated using the peak-area ratio of the analyte to internal standard using LCQuan software (Thermo Fisher Scientific, MA, USA).

Method validation. Validation of the assay was tested as adapted from European Medicines Agency guidelines on Bio-analytical Validation.[13] Specifically, we determined the lower limit of quantification (LLOQ), carryover, accuracy, linearity, precision, recovery and stability of the assay.

The LLOQ was defined as the lowest standard with a signal to noise ratio ≥ 5 and a signal for tofacitinib at least 5 times that of blank serum samples. Carryover was calculated as the percentage of residual signal present in a blank sample following an injection of 1000 ng ml^{-1} tofacitinib. 50 μl of serum was spiked with tofacitinib/tofacitinib-d3 to achieve final concentrations of 0.01 ng ml^{-1}, 0.05 ng ml^{-1}, 0.1 ng ml^{-1}, 0.5 ng ml^{-1}, 1 ng ml^{-1}, 5 ng ml^{-1}, 10 ng ml^{-1}, 50 ng ml^{-1}, 100 ng ml^{-1}, 500 ng ml^{-1} and 1000 ng ml^{-1}. Tofacitinib-d3 remained constant at 50 ng ml^{-1}. In each run blank serum samples were also analysed. Samples were prepared in triplicate.

Linearity was tested in independent triplicate samples. 50 μl of serum was spiked with tofacitinib/tofacitinib-d3 to achieve final concentrations of 0.01 ng ml^{-1}, 0.05 ng ml^{-1}, 0.1 ng ml^{-1}, 0.5 ng ml^{-1}, 1 ng ml^{-1}, 5 ng ml^{-1}, 10 ng ml^{-1}, 50 ng ml^{-1} and 100 ng ml^{-1}. Tofacitinib-d3 remained constant at 50 ng ml^{-1}.

Accuracy and precision (within-run) were tested on 5 samples at final concentrations of 0.1 ng ml^{-1}, 0.25 ng ml^{-1}, 2.5 ng ml^{-1} and 50 ng ml^{-1}. Accuracy and precision (between-run) were evaluated on samples at final concentrations of 0.1 ng ml^{-1}, 0.25 ng ml^{-1}, 2.5 ng ml^{-1} and 50 ng ml^{-1} across 3 runs with a fresh standard curve prepared for each run. Accuracy of the assay is reported as the calculated concentration of each sample as a percentage of the nominal concentration.

Proof of concept study. To investigate the ability of the assay to detect adherence, a random selection of serum samples ($n = 10$) of patients prescribed tofacitinib who self-report adherent behaviour from the Biologics in Rheumatoid Arthritis Genetics and Genomics Study Syndicate (BRAGGSS) were analysed (REC reference: 04/Q1403/37). Participants self-reported date and time of tofacitinib ingestion prior to venepuncture. Samples were analysed in triplicate.

Results and discussion

Lower limit of quantification and carryover

The LLOQ for tofacitinib was 0.1 ng ml^{-1} as this gave a signal to noise ratio of ≥5. Carryover of sample following injection of a 1000 ng ml^{-1} tofacitinib was <1% (Fig. 1).

Linearity

Linearity was assessed using linear least-squared regression analysis and demonstrated excellent concordance (Fig. 2).
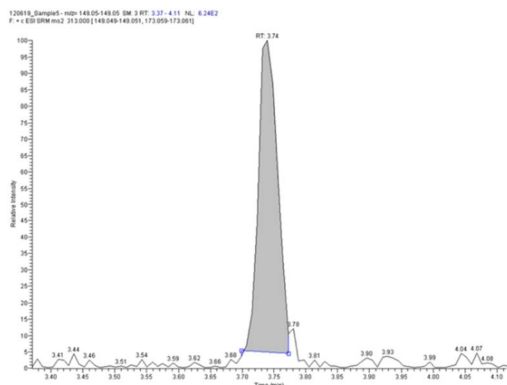
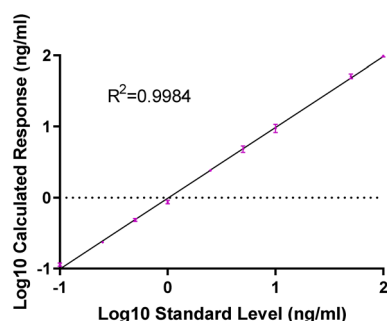**Fig. 1** Typical chromatogram obtained from a 0.1 ng ml⁻¹ tofacitinib injection.

Fig. 2 Results of the linearity experiment.

Accuracy and precision

Within run accuracy was between 81 and 85% at LLOQ and between 91 and 107% at all other levels. Between run accuracy was within 14.9% at LLOQ and within 0.2–5.1% of the nominal concentration at all other levels. Precision is reported as CV% either within run or between run at each concentration. Within run precision was 1.7–5.7% at LLOQ and 2.7–9.4% at all other concentrations. Between run precision was 3.4% at LLOQ and between 4.4 and 7.9% at all other concentrations (Table 1).

Recovery

Recovery was performed by comparing the measured concentration of tofacitinib in samples that were spiked with analyte before protein precipitation to samples that were protein precipitated and then spiked with the appropriate concentration on reconstitution in three separate runs similar to previous studies.[14,15] Tofacitinib was spiked at final concentrations of 0.1 ng ml⁻¹, 0.25 ng ml⁻¹, 0.5 ng ml⁻¹, 1 ng ml⁻¹, 2.5 ng ml⁻¹, 5 ml⁻¹, 10 ng ml⁻¹, 50 ng ml⁻¹ and 100 ng ml⁻¹. The internal standard tofacitinib-d3 remained at 50 ng ml⁻¹ in all samples. Recovery was calculated by dividing the response from the samples which were spiked prior to extraction by the response from the samples spiked after extraction. The mean recovery for tofacitinib ranged from 106% to 111% (mean 108%, Table 2).

Table 1 Tofacitinib assay accuracy and precision results

Nominal concentration (ng ml⁻¹)	Within run mean measured tofacitinib (ng ml⁻¹) (SD)	Within run mean accuracy (%) (SD)	Within run precision (%CV)	Between run mean measured tofacitinib (ng ml⁻¹) (SD)	Between run mean accuracy (%) (SD)	Between run precision (% CV)
0.1	0.12 (0.00)	81.5 (2.0)	5.7	0.117 (0.00)	85.1 (3.09)	3.43
0.25	0.27 (0.02)	93.3 (6.3)	9.4	0.264 (0.02)	94.9 (6.63)	6.88
2.5	2.28 (0.16)	108.8 (6.6)	7.2	2.4 (0.19)	104.1 (8.35)	7.91
50	50.6 (2.04)	98.8 (4.1)	3.7	50.1 (2.20)	99.8 (4.44)	4.41

Table 2 Tofacitinib recovery results

Expected concentration (ng ml⁻¹)	Spiked before extraction mean (ng l⁻¹) (SD)	Spiked post extraction mean (ng l⁻¹) (SD)	Mean tofacitinib recovery (%)
0.1	0.12 (0.02)	0.11 (0.01)	108.55
0.25	0.28 (0.01)	0.25 (0.01)	110.71
0.5	0.52 (0.04)	0.49 (0.04)	106.65
1	0.99 (0.08)	0.89 (0.03)	111.28
2.5	2.61 (0.13)	2.41 (0.16)	108.24
5	5.30 (0.22)	4.82 (0.06)	109.88
10	10.7 (0.75)	9.91 (0.34)	104.66
50	55.21 (3.83)	50.20 (0.82)	109.98
100	108.26 (4.57)	100.39 (0.47)	107.84

Table 3 Results of tofacitinib biochemical adherence testing

Sample ID	Time difference between self-reported tofacitinib ingestion and blood sample (hours)	Mean tofacitinib (ng ml⁻¹, $n = 3$)	CV (%)
49	7.0	26.43	2.82
40	2.8	100.55	5.51
70	3.6	73.23	2.27
68	1.5	150.37	8.09
20	1.5	137.46	9.09
30	1.8	117.85	14.97
33	1.4	90.89	1.18
79	5.4	217.47	11.40
17	2.8	108.43	7.43
37	2.5	335.04	3.48

Proof of concept study

To demonstrate the potential of the assay to biochemically measure adherence, all 10 BRAGGSS samples of tofacitinib treated RA patients successfully revealed tofacitinib levels above 0.1 ng ml⁻¹ with CV < 15% (Table 3).

Conclusions

Adherence to long-term medication is suboptimal and costs the NHS £500 million per year.[16] Non-adherence is a common health behaviour that is difficult to measure. Clinicians are often unaware of medication non-adherence and the development of an objective test of non-adherence is a vital first step so that supportive interventions can be put in place to optimise patient adherence.

We have successfully developed a novel sensitive tofacitinib LC-MS/MS assay with its sensitivity and validation against the EMA guidelines. Furthermore, the ability of the assay to measure circulating tofacitinib has been explored from patient samples and compared with self-reported adherence. Further research to investigate the ability of the assay to detect adherence in a heterogenous population prescribed tofacitinib using a population pharmacokinetic approach is warranted. This assay is appropriate for use in further research studies to establish the sensitivity of the assay for the detection of non-adherence.

Author contributions

SC formal analysis. KH conceptualization, methodology and data curation. KO methodology. RDU supervision, methodology and resources. AB conceptualization, methodology and data curation. JB conceptualization, methodology, funding acquisition. All authors writing – review & editing.

Conflicts of interest

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