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Therapeutic drug monitoring of tacrolimus by liquid chromatography–tandem mass spectrometry: Is it truly a routine test?☆,☆☆

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ABSTRACT

Therapeutic drug monitoring of tacrolimus by high-performance liquid chromatography–tandem mass spectrometry has become standard practice. We report on the long-term (4.5 years) use of one such method. Whole blood samples (25 μL) were treated with zinc sulphate (100 μL) and acetonitrile containing ascomycin (internal standard, 250 μL). A high-performance liquid chromatography–tandem mass spectrometer operating in positive ion mode with an electrospray interface was used. Chromatography was performed on a TDM C_{18} cartridge column (10 mm × 2.1 mm, 10 μm, Waters) using a switch gradient. A total of 4029 batches were analyzed for tacrolimus; this comprised of 81950 analyses of which 61027 were patient samples. Calibration curves (1.0–50 μg/L) were run on 1765 occasions (mean r^2 = 0.999; range r^2 = 0.988–0.999). Inter-batch accuracy and imprecision of the method (2.5, 12.5 and 30.0 μg/L), when in routine use, was 97.6–98.5% and <8.0%, respectively (n = 4031). Evaluation of the method against other methods in an external quality control scheme revealed good agreement by linear regression analysis (y = 0.924x + 0.196, r^2 = 0.985). The percentage difference between our results and that of all methods revealed a mean bias of −6.3% and a range of −33.3% to 11.1%. During the evaluation period, four batch failures occurred (0.1% failure rate) and greater than 1000 samples per analytical column was achieved. In conclusion, the described method is ideally suited as a routine test for tacrolimus in the clinical setting.

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1. Introduction

Tacrolimus is a potent immunosuppressant drug used for the prophylaxis of rejection in solid organ transplant recipients (Fig. 1). It is a critical dose drug and the role of therapeutic drug monitoring to optimise use is well established [1]. Elevated circulating tacrolimus concentrations can lead to serious toxicity and long term morbidity, while rejection can occur if a patient is under dosed with this drug. The individualisation of tacrolimus therapy through measured concentrations aims to achieve maximum therapeutic response with minimal adverse effects. The narrow therapeutic window of tacrolimus means that accurate measurement is an important aspect in effective treatment [2,3]. The current approaches for the measurement of tacrolimus are divided between two technologies; immunoassays and liquid chromatography–tandem mass spectrometry (LC–MS/MS) methods [4].

Immunoassays are highly suited to a routine laboratory with excellent automation and high throughput [5,6]. The major weakness of this technology is the non-specific binding of the antibody to tacrolimus metabolites and endogenous compounds [7–10]. In contrast, LC–MS/MS is highly specific for tacrolimus as it relies on the physicochemical properties of the analyte for detection and as such has become a very powerful analytical tool for the clinical laboratory [11,12]. The selectivity benefit of LC–MS/MS has encouraged some laboratories to adopt this technology for tacrolimus monitoring [13]. There have been several recent reviews on the use of LC–MS/MS for the monitoring of tacrolimus and the other immunosuppressants in this class of drugs [14–17].

The early tacrolimus LC–MS/MS methods, as published by our group and others, had extensive sample preparation; with protein precipitation followed by off-line solid phase extraction or liquid-liquid extraction [18–20]. These methods were developed to encompass a wide analytical range (typically, 0.2–100 μg/L) as, at the time, knowledge of tacrolimus therapeutic ranges were still evolving.

Improvements in technology have produced LC–MS/MS instruments with greater sensitivity. This increased sensitivity combined with a required analytical range of approximately 1–50 μg/L, has

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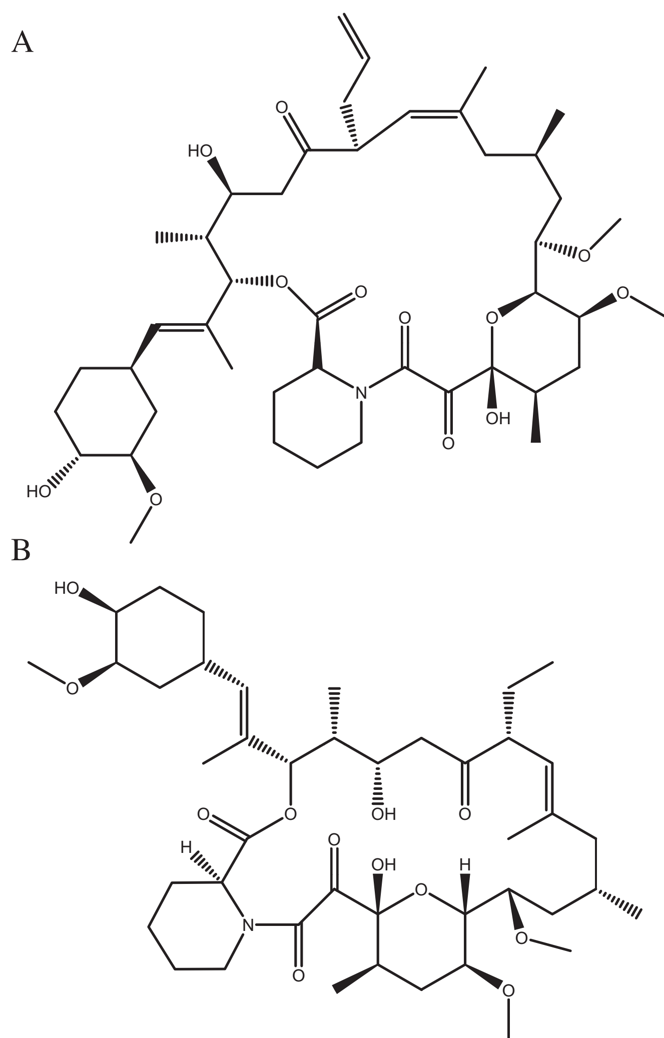

Fig. 1. The chemical structures of (A) tacrolimus and (B) ascomycin (internal standard).

allowed the simplification of methods. Two main approaches are currently employed for sample preparation; column-switching also known as 2-dimensional chromatography [21–24] and direct injection [25]. Both approaches would appear suitable for tacrolimus measurement.

While many studies have been published on the development and validation of tacrolimus LC–MS/MS methods, there has been no long-term evaluation of a LC–MS/MS method when used in a routine clinical setting. The aim of this paper is to report on the analytical performance of a tacrolimus LC–MS/MS method during five and half years of routine clinical use and to utilize these data to investigate the robustness and reliability of such methods.

2. Experimental

2.1. Chemicals and reagents

Tacrolimus and ascomycin (internal standard, Fig. 1) were a kind gift of Astellas Pharma (Tokyo, Japan). HPLC grade acetonitrile and methanol were purchased from Merck KGaA (Darmstadt, Germany). Ammonium acetate, formic acid and zinc sulphate were purchased from Sigma–Aldrich (St. Louis, MO, USA). Water was obtained from a Milli-Q water purification system (Millipore, Milford, MA, USA).

2.2. Calibrators and quality controls

In-house prepared calibration standards (1.0, 5.0, 10, 25, and 50 μg/L) and quality controls (2.5, 12.5, and 30 μg/L) were prepared in tacrolimus-free whole blood containing ethylenediaminetetraacetic acid as an additive. Calibration standards and quality controls were prepared from independent stock solutions. During routine use of the method, the first batch analyzed during a working day contained a 5-point standard curve and was processed along with 3 quality controls and a number of patient samples (range 1–62 patient samples). For subsequent batches only 3 quality controls were included for batch acceptance. Calibration standards and quality controls were randomly distributed throughout a batch. The number of batches analyzed during a working day varied between 1 and 5. Calibration curves were constructed using weighted $1/x$ linear regression (1.0–50 μg/L).

2.3. Liquid chromatography–tandem mass spectrometry

The LC–MS/MS system consisted of an Alliance HT LC system interfaced to a Quattro Micro tandem mass spectrometer by an electrospray ion source (Waters Corporation, Milford MA). The instrumentation was controlled by MassLynx software (V4.0, Waters). Chromatography was performed on a TDM C_{18} cartridge column (10 mm × 2.1 mm, 10 μm, Waters) which was maintained at 55 °C. The mobile phase consisted of (A) ammonium acetate (2 mmol/L) and formic acid (1 mL/L) in water and (B) ammonium acetate (2 mmol/L) and formic acid (1 mL/L) in methanol. A binary step gradient at a flow rate of 0.6 mL/min was employed. The gradient used was: 50% B as initial conditions; at 0.4 min a switch to 100% B; at 0.8 min a switch to 50% B. The mobile phase eluent was diverted to waste for the first 0.4 min of the 2 min chromatographic run.

Mass spectrometric detection was by selected reaction monitoring, with ions generated in the positive mode. The following mass transitions were monitored; tacrolimus m/z 821.3 → 768.3; internal standard m/z 809.3 → 756.3. A dwell time of 150 ms was used for each mass transition. The compound specific parameters were set to the following values: capillary voltage 1.0 kV; cone voltage 30 V; collision energy 22 eV. A source temperature of 140 °C; desolvation temperature of 350 °C and desolvation gas flow of 600 L/h were used to provide optimal ion generation.

2.4. Sample preparation

The tacrolimus LC–MS/MS assay used was based on the reported method of Keevil et al. [25]. Whole blood standards, controls and patient samples (25 μL) were treated with 0.1 M zinc sulphate (100 μL) in 2 mL polypropylene 96-round well plates and vortex mixed for 10 s. Acetonitrile containing ascomycin (5 μg/L; internal standard; 250 μL) was added. The plates were capped, vortexed for 2 min, centrifuged (3 min at 800 × g) and supernatant was injected (20 μL).

2.5. Analytical performance

An initial evaluation, before implementation for routine service, was performed on the tacrolimus LC–MS/MS method by measuring quality control samples in replicates of 5 on one day and in singlicate on each of 5 days. These data were used to determine inter- and intra-day accuracy and imprecision. The analytical performance of the method was assessed, during routine use, by a retrospective investigation of all clinical batches of patient samples that were analyzed from November 2004 to April 2010. Performance based on linearity was determined from calibrators run within batches. The inter-batch accuracy and inter-batch imprecision was calculated

from the internal quality controls analyzed within all batches. Robustness was evaluated, in terms of batch failures and number of samples analyzed per analytical column, during the study period.

Accuracy of the LC–MS/MS method was further assessed using the monthly results obtained by our method for external quality control samples (65 cycles) obtained from the Tacrolimus International Proficiency Testing Scheme (http://www.bioanalytics.co.uk/). There were 3 “results blinded” blood samples provided per cycle. Samples from this scheme included blood from patients not treated with tacrolimus, pooled patient samples and blood from patients not treated with tacrolimus but spiked with the drug. Our results were compared to the mean results obtained by the scheme for all liquid chromatography assays, using linear regression analysis and the methods described by Bland and Altman [26].

Matrix effects were evaluated by the post-column infusion method [27]. Tacrolimus (100 μg/L) was infused at a flow rate of 20 μL/min into the LC–MS/MS operating under the conditions of the method. The mass transition for tacrolimus was monitored. Water and blood (not containing tacrolimus) samples were prepared and analyzed as per the procedure described herein. Comparison of tacrolimus responses for the water and blood samples was undertaken. In addition, phospholipids were monitored for the blood sample using the mass transition (m/z 184.1 → 184.1) and mass spectrometric conditions described by Little et al. [28].

3. Results and discussion

A total of 4029 batches were analyzed for tacrolimus between November 2004 and April 2010. This comprised 81,950 analyses, of which 61,027 were patient samples. The remainder were calibrators and quality controls. The majority of samples analyzed were from patients, both pediatric and adult, who had undergone solid organ transplantation (i.e. renal, liver or heart). There were some samples from patients being treated with tacrolimus for autoimmune diseases.

While there is a growing acceptance for LC–MS/MS to be used in the clinical setting and in particular for the monitoring of tacrolimus, this is the first reported long-term evaluation of an LC–MS/MS method for the measurement of tacrolimus. Lensmeyer and Poquette [29] reported on the routine use of a liquid chromatography–mass spectrometry method over a 12 month period, but no analytical performance data were stated.

Representative chromatograms of blood samples from a patient not receiving tacrolimus and a renal transplant patient receiving tacrolimus therapy are shown in Fig. 2. The combination of protein precipitation for sample preparation in a 96-well format, once daily method calibration and the rapid chromatography of the switch gradient (3 min) approach provide sufficient throughput to analyse at least 100 patient samples per day. Throughput may be furthered improved if the injection cycle time was decreased, as this is currently 1 min of the total analysis. One possible limitation of the batch analysis approach is the delivery of results for urgent one-off tests. As such, for our method the flexibility of random access is not available.

Calibration curves were run on 1765 occasions (mean r^2 = 0.999; range r^2 = 0.988–0.999). The inter-batch accuracy and imprecision of the tacrolimus LC–MS/MS method determined from the back calculated calibrator results was 99.4–100.9% and < 6.2%, respectively (n = 1765). The linear range of the method (1.0–50 μg/L) would appear adequate for routine monitoring of trough samples and pharmacokinetic studies if required. However, the current trend is for lower doses of tacrolimus to be used in the maintenance of patients [30]. Therefore an improved lower limit of quantification may be required in the near future. With the ongoing improvements

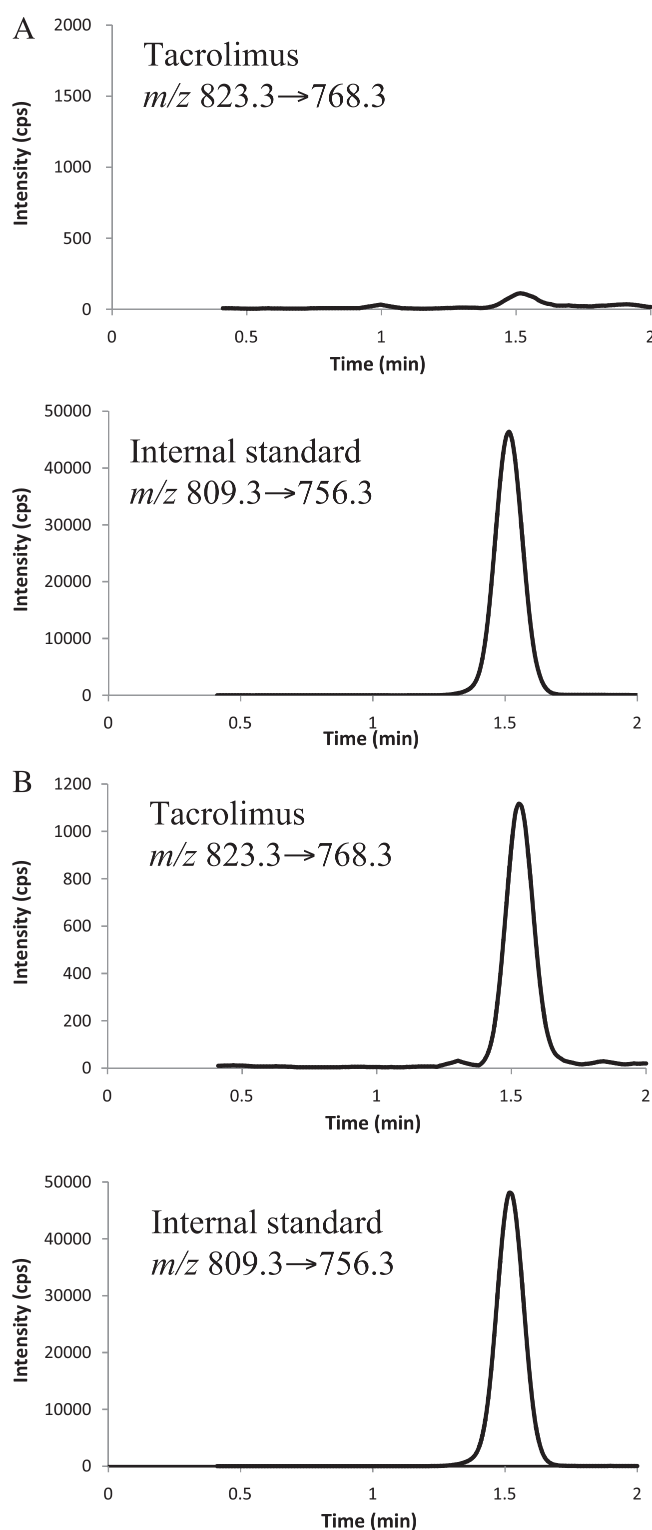

Fig. 2. Representative chromatograms of tacrolimus (m/z 823.3 → 768.3) and the internal standard (m/z 809.3 → 756.3) for blood samples obtained from (A) a patient not receiving tacrolimus therapy and (B) a 15 year old renal transplant recipient receiving tacrolimus therapy (1 mg bd; 3.3 μg/L).

in instrument sensitivity, the method reported in this study may be suitable for low dose monitoring, but using a more “high-end” LC–MS/MS. Another weakness of the described method is the lack of confirmatory mass transitions for tacrolimus and the internal standard. The use of confirmatory mass transitions has been recently advocated [31–33] and should be employed where possible. For the

Table 1
Accuracy and imprecision of the tacrolimus LC–MS/MS method assessed using quality controls during initial validation[#] (n = 5) and routine use* (n = 4031).

Tacrolimus concentration (μg/L)	Accuracy[a] [imprecision[b]] (%)		
	Intra-day[#]	Inter-day[#]	Inter-batch*
2.5	94.4 [2.8]	101.6 [4.5]	97.7 [7.9]
12.5	96.2 [0.9]	100.3 [4.0]	97.6 [5.1]
30	97.2 [2.4]	101.1 [2.5]	98.5 [4.9]

[a] Accuracy = mean measured concentration/nominal concentration × 100%.
[b] Imprecision, expressed as co-efficient of variation = standard deviation/mean measured concentration × 100%.

current method, we were unable to obtain the desired lower limit of quantification (1.0 μg/L) if secondary transitions were used. This was a limitation of the instrumentation employed.

The analytical performance of the method, in terms of quality control measurement, during routine use was acceptable when compared to initial validation data (Table 1) and according to the proposed guidelines of Viswanathan et al. [34]. Inter-batch accuracy and imprecision of the method when in routine use was 97.6–98.5% and <8.0%, respectively (n = 4031; Table 1). These data support the idea that once daily calibration of the tacrolimus method can be undertaken while still maintaining analytical performance. Once daily calibration can provide up to an extra 60 min of analysis time per day and as such assist in meeting clinical demands for rapid turnaround time of results.

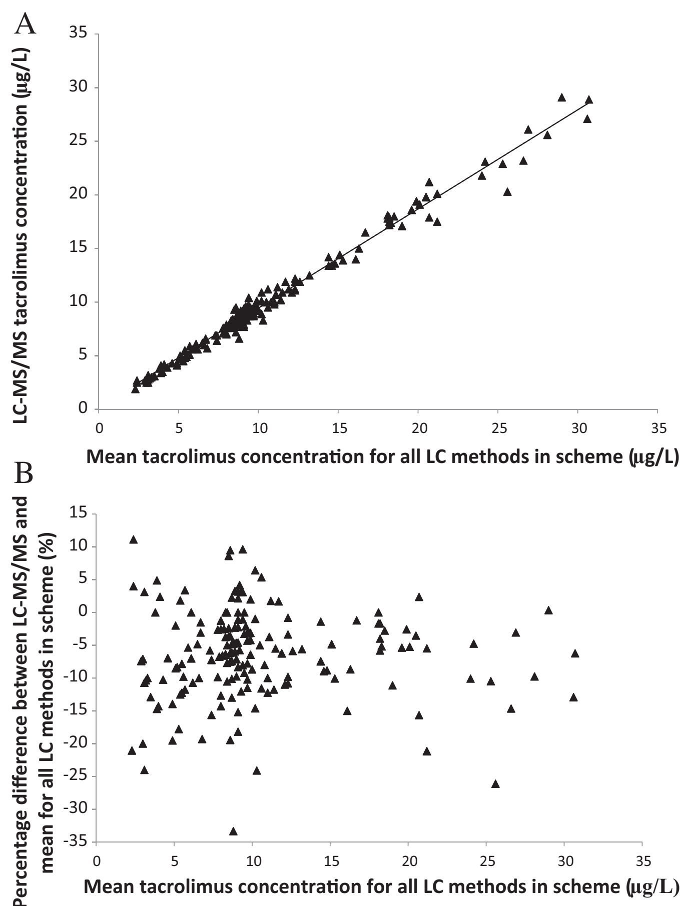

Fig. 3. A comparison of tacrolimus results obtained by our routine LC–MS/MS assay against all chromatographic methods in an external quality control scheme (n = 182). Comparisons were made using (A) linear regression analysis (r^2 = 0.985) and (B) the methods described by Bland and Altman (mean difference = −6.3%; range −33.3% to 11.1%) [26].

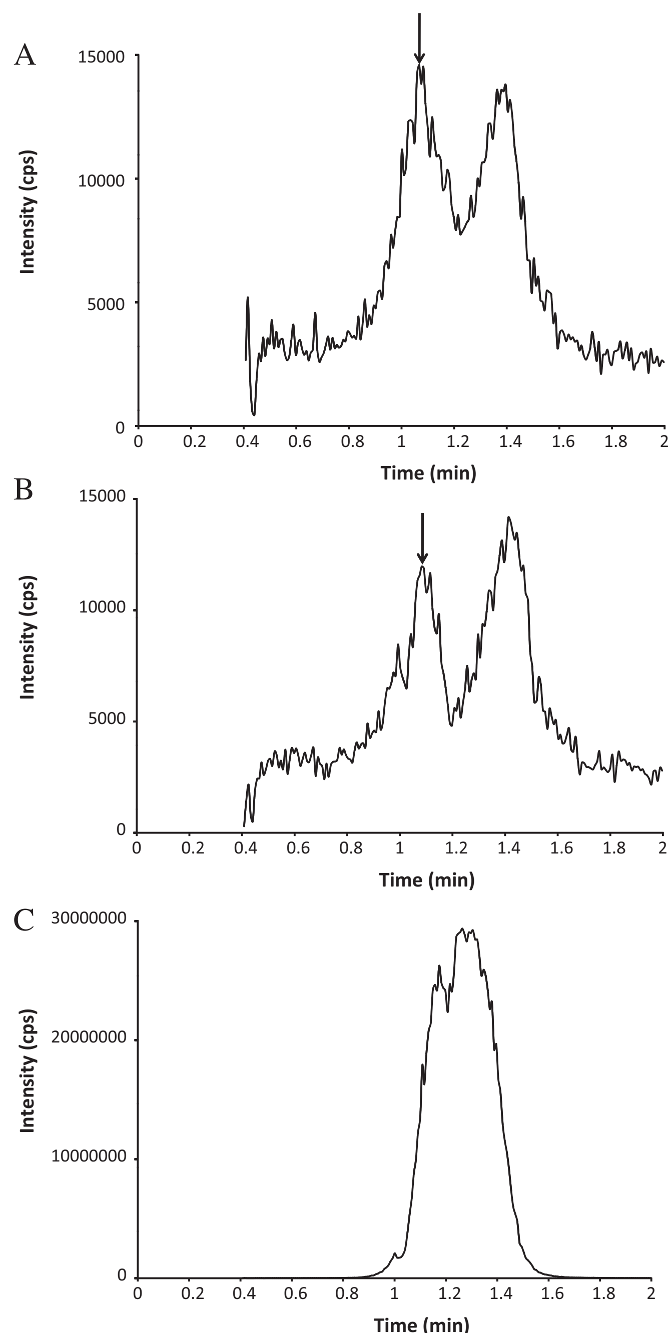

Fig. 4. Matrix effects investigated using post-column infusion studies (see Section 2.5 for details). Chromatograms represent (A) a water sample injected and (B) a whole blood extract injected. Phospholipids (C) were monitored using the using the mass transition (m/z 184.1 → 184.1) and the mass spectrometric conditions described by Little et al. [28].

A total of 195 external quality controls were analyzed over the evaluation period. Of these, 13 samples were pooled blood from patients not receiving tacrolimus. For all of these samples a result of less than the lower limit of quantification (1.0 μg/L) was reported. These data provide some evidence on the selectivity of the current method. The remaining 182 external quality controls contained tacrolimus; either pooled transplant patient samples (n = 65) or blood to which the drug had been added (n = 117). Comparison of these results against the mean results obtained by all liquid chromatography methods in the scheme is shown in Fig. 3. Linear regression analysis (Fig. 3A) showed good agreement between our results and the mean results (y = 0.924x + 0.196; r^2 = 0.985; n = 182).

The percentage difference between our results and that of all methods revealed a mean bias of −6.3% and a range of −33.3% to 11.1% (Fig. 3B). Examining only pooled transplant patient samples (renal $n = 59$; liver $n = 6$) showed a percentage difference between our results and that of all methods of −4.4% with a range of −19.4% to 9.6% ($n = 65$). The negative mean bias observed may be caused by a calibration difference between our method and others. This claim is supported by data from a recent study in which we reported an approximate bias of −10% bias for patient results when compared against a commercial tacrolimus kit that used certified calibrators [35]. These results highlight the potential for inaccuracy that may be associated with in-house prepared calibration material and the need for laboratories to subscribe to external proficiency testing schemes.

The results of matrix effect studies are shown in Fig. 4. The chromatograms for the water and blood samples exhibit similar response at the retention time of tacrolimus (1.08 min). The retention time of tacrolimus overlaps with the elution of endogenous phospholipids. Thus it would appear that there would be some suppression of tacrolimus signal due to co-eluting phospholipids. The excellent results obtained from our external quality control samples would suggest that the internal standard (ascomycin) is adequately compensating for these changes in response. A significant region of signal suppression is evident at a retention time of 1.2 min. This suppression is probably due to the large amount of phospholipids that were observed to elute at this time (Fig. 4C).

During the study period, the method was undertaken by four scientists. It should be stressed that the instrument used during this time was dedicated to one assay, tacrolimus. Maintenance, in the form of a source clean, was performed on a monthly basis. A yearly preventative maintenance procedure was undertaken by an engineer from the instrument manufacturer. Instrument downtime was limited to one board failure during the first fortnight of use and when maintenance was performed. From our experience it would be advisable to have a second mass spectrometer available for use during these periods. The robustness and reliability is illustrated by the incidence of only four batch failures out of the 4029 batches (0.1% failure rate). Of the four batch failures, three were due to errors attributed to the scientist and one due to instrument malfunction. The total number of analytical columns used over the evaluation period was 63, demonstrating the ability to analyze greater than 1000 samples per column. These data further suggest ruggedness in the method.

4. Conclusions

The LC–MS/MS method reported has been shown to be reliable and robust over a four and a half year period of routine use. The combination of rapid sample preparation and chromatography provides a method suited to the demands of a clinical service. We conclude that the described LC–MS/MS method can be truly considered a “routine” test for tacrolimus.

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