Comparison Between an Automated and Manual Extraction for the Determination of Immunosuppressive Drugs Whole Blood Concentrations by Liquid Chromatography Tandem Mass Spectrometry

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Background: The whole blood extraction for liquid chromatography tandem mass spectrometry (LC-MS/MS) of simultaneous quantification of cyclosporine A (Cys A), tacrolimus (Tacrs), sirolimus (Siros), and everolimus (Evers) is still performed manually in many laboratories. The analytical results obtained with an automated method using a liquid handler versus a classical manual preparation were compared. **Methods:** Cys A ($n = 36$), Tacrs ($n = 50$), Siros ($n = 34$), Evers ($n = 38$) whole blood samples of patients were analyzed by LC-MS/MS assay after manual preparation and automated process using a liquid handling platform including a centrifugation step. **Results:** The comparison

between manual and automated extraction investigated by a linear regression showed a high correlation between results [(Tacrs “automated”) = $1.0927 \times$ (Tacrs “manual”) – 0.36; (Cys A “automated”) = $1.0284 \times$ (Cys A “manual”) + 0.0312; (Siros “automated”) = $0.9923 \times$ (Siros “manual”) + 0.4001; (Evers “automated”) = $1.0000 \times$ (Evers “manual”) – 0.0600]. **Conclusion:** The results obtained by the automated and manual preparation are consistent. The automated method is applied for high-throughput therapeutic drug monitoring of immunosuppressive drugs in routine practice, leading to an increase in quality. J. Clin. Lab. Anal. 30:924–929, 2016. © 2016 Wiley Periodicals, Inc.

Key words: automation; cyclosporine A; tacrolimus; everolimus; sirolimus

INTRODUCTION

Therapeutic drug monitoring (TDM) of cyclosporine A (Cys A), tacrolimus (Tacrs), everolimus (Evers), and sirolimus (Siros) is recommended to optimize efficacy and minimize toxicity in individual patients. For this monitoring, the analysis of immunosuppressive in whole blood is performed either by immunoassays or by liquid chromatography/tandem mass spectrometry (LC-MS/MS). As drawbacks of immunoassays, analytical performances are limited in terms of sensitivity for some immunoassays or specificity due to cross-reactivity with metabolites and endogenous compounds, and reagent costs are relatively high in comparison with LC-MS/MS assays (1,2). However, LC-MS/MS methods required usually labor-intensive and time-consuming sample preparation steps. Moreover, LC-MS/MS assays are not easy to integrate into laboratory workflow due to the lack of connection

between laboratory information system and LC-MS/MS system. To our knowledge, two automated LC-MS/MS assays based on sample preparation by a liquid handling platform including a filtration step by vacuum (3) or associated with centrifugation (4) have been previously developed for Tacrs, Evers, and Siros (3) and Cys A, Tacrs, Evers, and Siros (4). No automated LC-MS/MS method based on preparation of samples including no filtration step has been published for a simultaneous monitoring of four immunosuppressive drugs, Cys A, Tacrs, Evers, and

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Received 14 December 2015; Accepted 2 February 2016

DOI 10.1002/jcla.21958

Published online in Wiley Online Library (wileyonlinelibrary.com).

Siros. The aim of this work is to compare the analytical results of this automated method and a classical manual sample preparation associated with an LC-MS/MS assay in the context of routine TDM of immunosuppressive drugs.

MATERIALS AND METHODS

Patients

All patient samples tested in this work were collected from an ongoing drug-monitoring program and were reported in accordance with ethical guidelines. Indeed an informed consent was not required. Whole blood levels of Cys A ($n = 36$), Tacrs ($n = 50$), Siros ($n = 34$), and Evers ($n = 38$) were obtained.

Chemicals and Reagents

Tacrs was kindly donated by Astellas-Pharma (Levallois-Peret, France), Evers and CysA by Novartis Pharma (Rueil-Malmaison, France), and Siros by Pfizer (Paris, France). Stable isotope-labeled internal standards (Tacrs- d_3 , Evers- d_4 , Siros- d_3 , CysA- d_{12}) were purchased from LGC (Toronto Research Chemical, North York, Canada). The protein precipitation solution was a mixture of methanol and 0.2 M $ZnSO_4$ (80:20, v/v) containing the four internal standards (Tacrs- d_3 , 10 ng/ml; Evers- d_4 , 15 ng/ml; Siros- d_3 , 20 ng/ml; CysA- d_{12} , 50 ng/ml). Calibrators and quality control samples were provided from Chromsystems Chemicals GmbH (Munich, Germany). All solvents and reagents were of HPLC-grade and were obtained from VWR International (Fontenay-sous-Bois, France).

Automated Samples Processing

The robotic system included an automated tube bar code reader, a liquid handling platform (Biomek NX^P, Beckman Coulter, Brea, CA) for homogenization, pipetting, distribution and mixing of plate by agitation, a robotic transport for transferring precipitation plate to centrifuge and back to Biomek NX^P, and centrifuge (Sigma 6–16 KL). The Biomek NX^P was equipped with eight individual pipettes (four with needles for piercing sample tubes and four using disposable tips), one robotic manipulator arm for plate movement, and an orbital shaker for 96-well plates. The robot was controlled by a computer using Beckman Biomek software 4.1.

The sample preparation by the automated liquid handler includes (a) identification of samples by sample tubes barcode reading (four sample tubes collected with K_3EDTA as anticoagulant per rack) for producing a sample list that is transferred to LC-MS/MS system; (b)

Fig. 1. Automated sample preparation.

homogenization of whole blood (six tilts, four sample tubes are homogenized simultaneously); (c) after wash step for piercing pipettes and primary tube cap piercing, pipetting of 100 μ l of samples 1 mm below the surface, the liquid surface is detected by conductivity (the lower limit for primary blood volume is 500 μ l); (d) distribution in 96 deep well precipitation plate; (e) addition of $ZnSO_4$ /methanol containing deuterated internal standards (200 μ l); (f) mixing of plate by vigorous orbital shaking (1,500 rpm, 1 min), and centrifugation of plate ($2,800 \times g$, 10 min); (g) pipetting of deproteinized supernatants (100 μ l); and (h) distribution in 96-well collection plate. The single manual step of the method is to transfer the well plate to the LC-MS/MS autosampler (Fig. 1).

Manual Samples Processing

After homogenization of whole blood patient sample (or calibrator or quality control), a volume of 100 μ l of sample was mixed with 200 μ l of precipitation solution in 1.5 ml polypropylene tubes. The mixture was vortex mixed for 10 min, and centrifuged for 10 min at $15,300 \times g$

TABLE 1. Timetable for Mobile-Phase Flow Rate

Isocratic pump		Binary pump	
Time (min)	Flow (ml/min)	Time (min)	Flow (ml/min)
0.00	0.55	0.00	0.5
		0.05	3
		0.50	3
		0.55	0.5
		0.95	0.5
		1.00	3
		1.45	3
1.50	0.55	1.5	0.5

at 4°C. Subsequently, the supernatant was transferred in a polypropylene tube with pierceable membrane screw caps.

LC-MS/MS

The chromatographic system consists of Agilent 1200 Series components (Palo Alto, CA), including a binary pump, isocratic pump, column oven, and auto-sampler. The hardware configuration included a Sciex API 3200 (Toronto, Canada) equipped with a turboionspray source. Two-dimensional LC was performed. Twenty-five microliters are injected in the chromatographic system. First dimension chromatography was an on-line enrichment and purification performed by a perfusion column (POROS R1/20, 2.1 × 30 mm, Applied Biosystems, Carlsbad, NM). The binary pump supplied methanol–water (20/80, v/v—3 ml/min for 0.45 min) for enrichment of analytes and internal standards on a Poros column. A back-flush elution was performed by the isocratic pump (methanol–water 97/3, v/v; 10 mM ammonium formate + formic acid [0.1%]) and a second dimension chromatography was performed using a Phenomenex Luna 5 µm Phenyl Hexyl, 2 × 50 mm column housed in an oven at 60°C. Chromatographic separation was performed by means of an appropriate flow program (Table 1). Positive ion mode MRM

transition was used for each drug as internal standards (Table 2). Dwell time was set at 60 msec for each compound. The Turboionspray interface settings and collision gas pressure were manually optimized (Ion spray voltage: 5500 V, temperature: 325°C, collision gas: 40.0 psi, curtain gas: 50.0 psi). MS/MS was performed with nitrogen as a collision gas. The 6-point calibration curves are calculated (area ratio using the internal standard vs. nominal concentration) and fitted by a linear least-squares regression with a weighting factor of 1/*x*. All the procedure was performed using the Analyst 1.5.2 software package.

Statistical Analysis

The comparisons of immunosuppressive drugs concentrations of patients obtained after automated versus manual extraction were performed using Passing–Bablok non-parametric linear regression and Bland–Altman plots.

RESULTS

The LC-MS/MS method allows a simultaneous quantification of Tacrs, Siros, Evers, and Cys A at a run-time of 1.5 min, providing a workflow of 30 samples /hr. The time to prepare 30 samples is consistent between manual and automated preparation (about 35 min). The equations of the regression line [(Tacrs “automated”) = 1.0927 × (Tacrs “manual”) – 0.36; (Cys A “automated”) = 1.0284 × (Cys A “manual”) + 0.0312; (Siros “automated”) = 0.9923 × (Siros “manual”) + 0.4001; (Evers “automated”) = 1.0000 × (Evers “manual”) – 0.0600] were close to the equation of the identity line (Fig. 2). The statistical differences between “automated” and “manual” results for the four immunosuppressive drugs were not significant because the 95% confidence interval for the intercept (Tacrs [–1.14 to 0.18], Cys [–10.53 to 6.93], Siros [–0.40 to 0.68], Evers [–0.33 to 0.21]) contains the value 0 and the 95% confidence interval for the slope (Tacrs [1.00–1.19], Cys [0.97–1.09], Siros [0.95–1.04],

TABLE 2. Settings for Mass/Charge (*m/z*) Transitions for Cyclosporine A (Cys A), Tacrolimus (Tacrs), Everolimus (Evers), and sirolimus (Siros) and Deuterated Internal Standards

Compounds	Precursor ion (<i>m/z</i>)	Product ion (<i>m/z</i>)	RT (min)	DP (V)	EP (V)	CEP (V)	CE (V)	CXP (V)
Cys A	1,202.9	425.4	1.3	125	8	48	70	16
CysA-d ₁₂	1,214.9	437.4	1.3	116	10.5	48	76	8
Evers	975.5	908.5	1.2	50	8	30	33	20
Evers-d ₄	979.5	912.5	1.2	50	8	30	33	20
Siros	931.5	864.5	1.2	50	8	30	33	18
Siros-d ₃	934.5	864.5	1.2	50	8	30	33	18
Tacrs	821.4	768.3	1.2	50	8	28	33	18
Tacrs-d ₃	824.4	771.3	1.2	50	8	28	33	18

RT, retention times; DP, declustering potential; EP, entrance potential; CEP, cell entrance potential; CE, collision energy; CXP, cell exit potential for API 3200 and the phenyl–hexyl column.

Fig. 2. Methods comparison. The Passing and Bablok regression plot (—) of tacrolimus (a; $n = 50$) sirolimus (c; $n = 34$), everolimus (e; $n = 38$), and cyclosporine A (g; $n = 36$) samples measured after automated or manual extraction and quantified by LC-MS/MS versus the identity line (---). Bland and Altman plot for tacrolimus (b, $n = 50$), sirolimus (d; $n = 34$), everolimus(f; $n = 38$), and cyclosporine A (h; $n = 36$) extracted by the automated versus manual method and quantified by LC-MS/MS. The ratio of concentrations obtained after automated and manual extraction is plotted against the mean value obtained after automated and manual extraction. The mean ratio is displayed by a bold solid line. Dotted lines represent mean ± 1.96 standard deviation of ratios.

Fig. 2. Continued.

Evers [0.95–1.05]) contains the value 1. The Bland and Altman plot between the two pretreatments yielded an average ratio (“automated” concentration/“manual” concentration) close to 1: respectively, 0.99, 1.01, 1.025, and 1.046 for Evers, Siros, Cys A, and Tacrs (Fig. 2). All the ratios of concentrations are included in the range, mean ratio ± 1.96 times the standard deviation of ratios, except one point for everolimus (ratio = 1.24, “automated” concentration = 3.1 ng/ml and “manual” concentration = 2.5 ng/ml). However, the difference between these values is not clinically relevant.

DISCUSSION

LC-MS/MS monitoring of immunosuppressant drugs has been developed to a large extent over the last few years (5). However, the sample preparation for the LC-MS/MS assays is still performed manually in many laboratories. Previous methods for preparing whole blood samples using robotic system coupled with LC-MS were developed in toxicology (6, 7) and TDM of immunosuppressive drugs (3, 4, 8). Contrary to these previous methods applied to TDM (3, 8), our method allows a simultaneous monitoring of Cys A, Tacrs, Evers, and Siros (vs. Tacrs (8) or Tacrs, Siros, and Evers (3)). The only manual action is the transfer of the deep-well plate from the robotic system to the LC-MS/MS autosampler that decreases the risk of sample misplacement during the preparation procedure while reducing musculoskeletal ailments of the technical staff (9). The manual decapping of the tubes (3, 4) was replaced by primary tube cap piercing that reduces the risk of exposure for the technical staff. To ensure the homogeneity of the whole blood sample, which is challenging as blood will quickly settle down, an original mixing procedure by agitation of blood collection

tubes was adopted instead of resuspension procedures by aspiration/dispensation (3, 4). The transfer of the deep-well plate from the robotic system to a centrifuge was done by a robotic manipulator arm thereby improving the automation of the process. During the extraction, the centrifugation without filtration was chosen after protein precipitation instead of filtration by vacuum (3) or filtration associated with centrifugation (4), thereby reducing the reagent cost since filter plates are usually more expensive than the deep-well precipitation plate used in our assay, while avoiding the risk of filter plugging. No decrease in analytical performance was observed with the automation and results are similar after manual or automated preparation for the four immunosuppressive drugs in patients. In 10 months (17,118 samples), the failures due to barcode reading (0.006 per 100 samples) or clots (0.02 per 100 samples) were rare. The submission of results to the laboratory information system after technical validation of results is automated by the connection between the software that controls the LC-MS/MS system and the laboratory information system without requiring manual input of results in the laboratory information system, which limits operator errors. The workflow (30 samples/hr) is limited by LC-MS/MS run-time and the automated preparation did not increase or decrease the throughput in comparison with the manual preparation. Because of the short run-time (1.5 min vs. 2 min (3)), the workflow was increased in comparison with the automated previously published LC-MS/MS method (30 vs. 19 samples/h(3)). This workflow is adapted for routine high-throughput TDM of immunosuppressive drugs (over 100 samples daily) because the typical overall total turnaround time is less than 5 h.

The analytical results of the automated assay in comparison with the classical manual results indicate that

this automated assay is an alternative method to the classical manual sample preparation. This automated method is particularly applied for high-throughput TDM of immunosuppressive drugs in routine practice, leading to safer sample handling and good ergonomics for the technical staff.

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