A new approach for the determination of immunosuppressive drugs using HPLC-MS/MS and Cs$^+$ adducts

Neuer Ansatz zur Bestimmung von Immunsuppressiva unter Einsatz von HPLC-MS/MS und Cs$^+$-Addukten

Abstract

In this study a new principle of measurement in LC-MS/MS (liquid chromatography mass spectrometry) for determination of the immunosuppressive drugs sirolimus, everolimus, tacrolimus, and cyclosporin A has been introduced by using the Cs$^+$ ion as the product ion in the multiple reaction monitoring mode (MRM). Separation of the immunosuppressive agents was achieved using a phenyl-hexyl-RP column together with a ternary gradient elution profile, consisting of water, methanol and acetonitrile combined with 0.1% v/v formic acid and 0.1 mmol/l Cs$^+$. Quantification was performed using cyclosporin D, ascomycin and 32-desmethoxy-rapamycin as internal standards. The inter-run precision of this new method, expressed as the coefficient of variation, was 2.57% for sirolimus, 2.11% for everolimus, 2.31% for tacrolimus and 2.11% for cyclosporin A.

Keywords: immunosuppressive drugs, LC-MS/MS, Cs$^+$ adducts

Introduction

The therapeutic drug monitoring (TDM) of immunosuppressive drugs in blood of organ-transplanted patients is of utmost importance to prevent intoxication or rejection due to incorrect dosage. Commonly used immunosuppressive agents are calcineurin inhibitors such as cyclosporin A and tacrolimus or the mTOR inhibitors sirolimus and everolimus. Recently a new therapeutic strategy has been to combine immunosuppressive drugs with different mechanisms of action in order to reduce undesirable side effects. This often leads to a lowering of the therapeutic concentrations of these drugs. Analytical problems occur in the detection of clinically relevant low levels of immunosuppressive agents, which in turn requires a more sensitive analytical measurement procedure at low analyte concentrations for all the drugs used. Several LC-MS/MS methods for routine measurement of immunosuppressive drugs in whole blood have been described [1], [2], [3]. These routine methods have been...
designed for high throughput combined with a short turnaround time. Methods without chromatographic separation of the analytes require cycles of only a few minutes per sample. Unfortunately, co-elution of the compounds usually leads to a bias in the analytical results, due to a lack of specificity of the mass transition in the MRM mode of the LC-MS/MS measurements [4], [5]. Avoidance of this phenomenon requires separation and detection of the parent substances from the different metabolites with biological activity.

LC-MS/MS procedures for determination of serum levels of digoxin and digitoxin have been recently published from this laboratory [6], [7] using Cs⁺ adduct formation, which allows potential improvement of both analytical sensitivity and specificity. The aim of the present study was to develop a method for determination of immunosuppressive agents, by utilising the principle of measurement of Cs⁺ adducts, thus forming the basis of a reliable method for defining target values for immunosuppressive drugs in external quality assessment schemes (EQAS).

This article describes a LC-MS/MS method with complete chromatographic separation of the immunosuppressive drugs cyclosporin A (cyclosporin A, Sandimmune<sup>®</sup>), tacrolimus (FK-506, Prograf<sup>®</sup>), sirolimus (rapamycin, Rapamune<sup>®</sup>) and everolimus (Certican<sup>®</sup>), with cyclosporin D, ascomycin and 32-desmethoxy-rapamycin as internal standards.

Materials and methods

Chemicals

Rapamycin (>99%), tacrolimus (FK-506, >99%) and cyclosporin A (>99%) were obtained from LC Laboratories, Woburn, MA, USA. Cyclosporin D and everolimus were a kind gift from Recipe, München, Germany. 32-desmethoxy-rapamycin was a kind donation from Wyatt, St. Davids, PA, USA. Ascomycin, formic acid (98%, puriss. p.a.) and cesium hydroxide (purity 99.97%), were purchased from Sigma-Aldrich, Taufkirchen, Germany. Methanol (LiChrosolv) was obtained from Merck, Darmstadt, Germany. Water was prepared using the purification system Direct-Q™ 5 (Millipore GmbH, Eschborn, Germany).

Apparatus

The HPLC system used was from Shimadzu (Duisburg, Germany) consisting of a SCL-10A system controller, three LC-10ADvp pumps (A, B, C), a DGU-14A degasser; a SIL-10AD autoinjector; a CTU-10AS column oven; and a FCU-12A Flow Switch (rotary valve). The Mass Spectrometer was a 4000 Q Trap equipped with a TurboV™ ESI source with Turboion Spray™ probe from Applied Biosystems (MDS-Sciex, Darmstadt, Germany).

HPLC-MS/MS measurement conditions

The chromatographic separation was performed on a phenyl-hexyl-RP column (Luna<sup>®</sup>, 2 x 150 mm, 5 µm, Phenomenex, Aschaffenburg, Germany). A ternary gradient was used for elution consisting of eluent A (pump A): 0.1% v/v HCOOH in water + 0.1 mmol/l CsOOCH; eluent B (pump B): 0.1% v/v HCOOH in methanol + 0.1 mmol/l CsOOCH and eluent C (pump C): 0.1% v/v HCOOH in CH₃CN + 0.1 mmol/l CsOOCH. CsOOCH was produced by neutralising CsOH with HCOOH to pH 7. The flow rate was 300 µl/min. The column temperature was set at 50°C. In order to avoid contamination of the ESI source a switching valve system (rotary valve A) was introduced. At position 0 of rotary valve A the eluate is passed into the ESI source. At position 1 the eluate is directed into waste (Figure 1, Table 1).

Table 1: Gradient profile for HPLC separation in detail

| Time  | Module   | Events         | Parameter |
|-------|----------|----------------|-----------|
| 0.01  | pumps    | % B            | 50        |
| 0.02  | subcontroller | rotary valve A | 1         |
| 1.00  | pumps    | % B            | 78        |
| 6.00  | subcontroller | rotary valve A | 0         |
| 16.00 | pumps    | % B            | 78        |
| 16.05 | pumps    | % C            | 0         |
| 20.00 | pumps    | % B            | 33        |
| 20.01 | pumps    | % C            | 39        |
| 30.05 | pumps    | % B            | 33        |
| 30.10 | pumps    | % C            | 39        |
| 31.00 | pumps    | % C            | 100       |
| 37.05 | pumps    | % C            | 100       |
| 37.10 | pumps    | % B            | 0         |
| 38.00 | pumps    | % C            | 0         |
| 38.05 | pumps    | % C            | 0         |
| 38.05 | pumps    | % B            | 50        |
| 42.00 | controller | Stop           |           |

The MS detection was performed in the positive ion mode. The mass transitions (m/z) for MS detection are given in Table 2. The m/z of 132.9 amu is the mass of the Cs⁺ ion.

Table 2: Mass transitions of the four immunosuppressive agents tacrolimus, sirolimus, everolimus and cyclosporin A and the internal standards ascomycin, 32-desmethoxy-rapamycin and cyclosporin D

| analyte           |precursor ion m/z [amu] |product ion m/z [amu] |
|-------------------|------------------------|-----------------------|
| tacrolimus        | 936.9                  |132.9                  |
| sirolimus         | 1046.8                 |132.9                  |
| everolimus        | 1090.8                 |132.9                  |
| cyclosporin A     | 1335.0                 |132.9                  |
| ascomycin         | 924.9                  |132.9                  |
| 32-desmethoxy-rapamycin | 1016.5        |132.9                  |
| cyclosporin D     | 1349.2                 |132.9                  |

The settings of the mass spectrometer are given in Table 3. (The abbreviations for settings are specific to the 4000 Q TRAP software used to control the system.)
Results

As shown in previous publications [6], [7] the generation of Cs⁺ ion adducts combined with LC-MS/MS offered great advantages in analysis of digoxin and digitoxin, both in SIM and in MRM mode. It was observed, that in the MRM mode, the Cs⁺ adduct as parent ion decomposes in a way that the Cs⁺ ion becomes detectable as product ion. This principle of measurement was investigated for the immunosuppressive agents tacrolimus, sirolimus, everolimus, cyclosporin A together with the analogues ascomycin, 32-desmethoxy-rapamycin and cyclosporin D used as internal standards.

The generation of Cs⁺ adducts is exemplified for cyclosporin A in Figure 2. The Q1 scan was performed by using a standard solution, dissolved in eluent B, which contained 0.1 mM Cs⁺. Under these conditions the singly charged Cs⁺ adduct generates the major signal (1335 amu). Only small amounts of the protonated form (1203 amu), the Na⁺ adduct (1225 amu) and the K⁺ adduct (1241 amu) are visible. Na⁺ and K⁺ probably originate from the manufacturing procedure and can be separated by HPLC.

Fragmentation of the cyclosporin A Cs⁺ adduct was studied using an 4000 Q TRAP in the enhanced product ion scan mode (Figure 3). The ion trap option offers a high mass resolution. In the product ion scan of Cs⁺ adduct of cyclosporin A with a parent mass of 1335 amu, the mass of 132.9 amu is the main signal. The isotope pattern of this signal and the defined mass indicates that this signal originates solely from Cs⁺.

During the mass transition the mass of 1202 amu is lost (Figure 4). This value corresponds to the mass of the unprotonated cyclosporin A molecule. Thus, it can be considered, that cyclosporin A is split off in form of a neutral molecule in the fragmentation process. This concept is supported by the results of the neutral loss scan. This scan demonstrates, that the only source of the neutral loss of the unprotonated cyclosporin A molecule is the Cs⁺ adduct of cyclosporin A with the mass of 1235 amu.

The adduct formation with Cs⁺ and the subsequent collision induced dissociation with Cs⁺ as the product ion was observed for all seven analytes. This principle of detection was used for the development of the chromatographic separation of all immunosuppressive drugs. A baseline separation was realized by using a ternary gradient (Figure 5).

In Figures 6-12 chromatograms of standard solutions of tacrolimus (Figure 6), sirolimus (Figure 7), everolimus (Figure 8), cyclosporin A (Figure 9) and the analogues cyclosporin D (Figure 10), ascomycin (Figure 11) and 32-desmethoxy-rapamycin (Figure 12) are shown in separate runs. In addition to the declared components, these standard solutions contain additional substances (Figures 6-10), which are separated during the chromatographic procedure. Tacrolimus, sirolimus, everolimus, ascomycin and 32-desmethoxy-rapamycin contain isobaric components, which probably represent isomers, as has been described elsewhere [8]. For the cyclosporins A and D cross contamination is observed (Figures 11-12). Cyclosporin A contains about 5% cyclosporin D and cyclosporin D contains about 1% cyclosporin A. This is of importance, as cyclosporin D is used as internal standard for quantification of cyclosporin A.

### Table 3: Instrument settings in the 4000 Q TRAP for MRM mode

| Event (program abbreviation) | Setting |
|-----------------------------|---------|
| CAD                         | medium  |
| CUR                         | 15.00   |
| GS1                         | 60.00   |
| GS2                         | 30.00   |
| IS                          | 5500.00 |
| TEM                         | 350.00  |
| ihe                         | ON      |
| DP                          | 140.00  |
| EP                          | 10.00   |
| CE                          | 100.00  |
| CXP                         | 14.00   |
Figure 2: Q1 scan of a cyclosporin A standard solution (1 µg/ml in eluent B) infused at a flow rate of 10 µl/min

Figure 3: Enhanced product ion scan of a cyclosporin A standard solution (1 µg/ml in eluent B) infused at a flow rate of 10 µl/min
Figure 4: Neutral loss scan of mass 1202 amu (1 µg/ml in eluent B) infused at a flow rate of 10 µl/min

Figure 5: HPLC-MS/MS chromatogram (overlaid graphs of single mass traces) of a standard mixture of tacrolimus (8.59 min), sirolimus (11.59 min), everolimus (12.70 min), cyclosporin A (24.12 min) and cyclosporin D (27.16 min), 10 µl (1 µg/ml) injected (for details see Table 1, Figure 1)
Figure 6: HPLC-MS/MS chromatogram (overlaid graphs of mass traces defined in Table 2) of a tacrolimus standard solution, 10 µl (1 µg/ml)

Figure 7: HPLC-MS/MS chromatogram (overlaid graphs of mass traces defined in Table 2) of a sirolimus standard solution, 10 µl (1 µg/ml)
Figure 8: HPLC-MS/MS chromatogram (overlaid graphs of mass traces defined in Table 2) of an everolimus standard solution, 10 µl (1 µg/ml)
Figure 9: HPLC-MS/MS chromatogram of a cyclosporin A standard solution, 10 µl (1 µg/ml)

upper panel: overlaid graphs of mass traces defined in Table 2
lower panel: extracted mass trace of cyclosporin D
Figure 10: HPLC-MS/MS chromatogram of a cyclosporin D standard solution, 10 µl (1 µg/ml)
upper panel: overlaid graphs of mass traces defined in Table 2
lower panel: extracted mass trace of cyclosporin A
For quantification of tacrolimus, ascomycin was used as internal standard, for sirolimus and everolimus the internal standard used was 32-desmethoxy-rapamycin.

The validity of this method was tested during the course of this study, in which the inter-run precision was measured using standard solutions. The precision measured — expressed as the coefficient of variation (CV) — under these conditions was 2.57% for sirolimus, 2.11% for everolimus, 2.31% for tacrolimus and 2.11% for cyclosporin A (Table 4).
Discussion

Recent LC-MS/MS measurements of immunosuppressive drugs have often used NH₄⁺ adducts, which give rise to several different product ions [4]. Addition of Cs⁺ ions reduced the number of product ions to one single product ion, namely the Cs⁺ ion, which is measurable with high specificity and high signal intensity. It is of utmost importance to have a highly specific analytical method with high accuracy and precision for use as an intended reference measurement procedure. The specificity of current LC-MS/MS methods should be improved by chromatographic separation [9]. In the method presented here, all four immunosuppressive drugs are fully separated. Different internal standards listed in the text have been used for the quantification of the four immunosuppressive drugs. The best results were obtained using the corresponding analogues, namely ascomycin for tacrolimus, 32-desmethoxy-rapamycin for sirolimus and everolimus and cyclosporin D for cyclosporin A. This has been discussed elsewhere by Vogeser et al [4].

A baseline separation of cyclosporin D from cyclosporin A has been realized, as well as the separation of sirolimus from 32-desmethoxy-rapamycin. If needed, separation of everolimus from the internal standard 32-desmethoxy-rapamycin or tacrolimus from the internal standard ascomycin can be made by slight modifications of the ternary gradient profile.

Until now, there are no pure reference materials available for the immunosuppressive agents. All analytes used have been extracted from biological sources and contain sodium and potassium ions (see Figure 2) which can be removed by chromatographic separation so that the presence of Na⁺ and K⁺ adducts was less than 2%.

The high throughput routine methods which have been published for determination of immunosuppressive drugs do not separate the different immunosuppressive agents chromatographically. As a result, the routine methods currently in use are not suitable for setting target values in EQAS. They have limited specificity [4] and are prone to mass interferences [5]. In patient samples problems may occur during quantification, due to the presence of metabolites. At least 13 metabolites have been described for cyclosporin A [10]. The metabolites are usually more hydrophilic than the parent substance, due to hydroxylation, and can be separated by modifying the ternary gradient system used here.

For optimal quantification it is desirable to have stable isotope labelled analytes as internal standards [11]. Unfortunately, at present there are no commercial sources for isotopically labelled immunosuppressive agents. When stable-labelled compounds become available the method presented here can be developed into a reference measurement procedure for immunosuppressive drugs.

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**Please cite as**
Kaiser P, Akerboom T, Wood WG, Reinauer H. A new approach for the determination of immunosuppressive drugs using HPLC-MS/MS and Cs⁺ adducts. GMS Ger Med Sci. 2005;4:Doc01.

**This article is freely available from**
http://www.egms.de/en/gms/2006-4/000030.shtml

**Received:** 2005-12-21
**Published:** 2006-01-18

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