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Article

An Experimental Approach To Risk Of Organ Rejection: Demonstration Of False Immunosuppressant Results Due To Radiopaque Agents

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Abstract:

Background: Immunosuppressant blood levels should be measured at regular periods in order to keep them within the therapeutic index. Although LC-MS/MS is preferred as a reliable method, some molecules like radiopaque agents in blood matrix may lead to false results. The aim of this study is to investigate the effect of seven different radiopaque agents on immunosuppressant drugs.

Methods: Seven different radiopaque agents were added into control materials containing tacrolimus, everolimus, sirolimus and cyclosporine A drugs. Measurements were performed by LC-MS/MS instrument. The amount of deviations from target values were calculated.

Results: Immunosuppressant blood levels significantly changed after the administration of radiopaque agents. Seven different radiopaque products led to false negative results in tacrolimus and cyclosporine A levels at a rate of 19.77% to 44.45%. The smallest deviations were seen in everolimus levels with administration of RM6 (gadodiamide) and in sirolimus levels with RM1 (gadobutrol) at rates of 4.04% and 2.11%, respectively. The highest deviations were observed with RM3 (iohexol) administration in everolimus and sirolimus levels at rates of 153.72% and 171.41%, respectively.

Conclusions: False immunosuppressant results associated with radiopaque agents may result in organ rejection. Preferring radiopaque agents that cause the least interference risk is important to reduce the organ rejection risk. However, the least risky method is to obtain samples for drug levels before contrast-enhanced imaging.

Keywords: Organ transplantation, immunosuppressant, radiopaque agents, interference

1. Introduction

Organ transplantation is the only treatment modality for patients with terminal stage organ failure [1]. Immunosuppressants are used to prevent rejection of the organ transplantation [2,3]. Blood levels should be measured at regular periods in order to keep these oral drugs within the therapeutic index [3]. LC-MS/MS reference method is used for these drugs since immunoassay methods used commonly may be affected by several endogenous and exogenous molecules [4,5].

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Although LC-MS/MS is a reference method, it may be influenced by the molecules in the matrix [6]. Among these molecules are radiopaque agents used during contrast-enhanced imaging. Macromolecular radiopaque agents that do not permit transmission of X-rays may interfere with immunosuppressant levels. The matrix effect produced by the impact of some molecules found in the blood on ionization phase of the LC-MS/MS measurement method results in false measurement of analytes [7,8]. This was initially demonstrated by Tang and Kebarle (1993) who showed that electrospraying reactions of analytes were reduced by increasing concentrations of organic bases. Although the mechanism underlying the matrix effect is unknown, it is likely to be caused by undetectable serum components that bind to an analyte [9]. False measurement of immunosuppressant concentration may lead to incorrect dose restriction or escalation. In particular, incorrect measurement of drug levels used for immunosuppression in liver or kidney transplantation poses a substantial risk for organ rejection [10,11]. This kind of interference is unpredictable. The focus of this experimental study is to investigate how immunosuppressant drug levels are influenced by radiopaque agents

2. Methods

2.1. Materials:

Six different levels of immunosuppressant calibrator (Jasem, Turkey, Lot: CL-3000420150616) and single-level control solution (Lot: CL-3000620150616) were used in this study. Radiopaque agents used for the interference study included iohexol (omnipaque, 755 mg/mL, 100 mL for intravenous injection, GE Healthcare), gadopentetate dimeglumine salt (emaray, 469.01 mg/mL, 15 mL solution for IV injection), gadodiamide (gadotu, 287 mg/mL, 15 mL solution for IV injection), ioversol (optiray, 741 mg/mL, 100 mL for intravenous injection), iohexol (kopaq, 755 mg/mL, 100 mL for intravenous injection), gadobutrol (gadovist, 604.72 mg/mL, 15 mL solution for IV injection), gadodiamide (gadodiem, 287 mg/mL, 15 mL IV solution for IV injection). All the solvents and reagents of HPLC were produced by JASEM.

2.2. Measurement Devices:

LC–MS/MS analyzes of the tacrolimus, sirolimus, everolimus, cyclosporine A compounds were performed by using a UHPLC (Nexera, Shimadzu X2, Japan) and a tandem MS instrument (Shimadzu 8045, Japan). The liquid chromatography was equipped with LC-40AD binary pumps. The chromatographic separation was performed on a immunsupresant analytical column (JASEM). The column temperature was fixed at 40 ºC. The elution gradient consisted of mobile phase A (water, 5 mM ammonium formate and 0.1% formic acid) and mobile phase B (methanol, 5 mM ammonium formate and 0.1% formic acid).. The solvent flow rate was maintained at 0.5 mL/min and injection volume was settled as 4 µl. For MS detection was carried out by Shimadzu LCMS 8045 model triple quadrupole mass spectrometer equipped with an ESI source operating in negative ionization modes. LC–MS/MS data were collected and processed by Lab Solutions software (Shimadzu, Kyoto, Japan). The multiple reaction monitoring (MRM) modes were used to quantify the analyzes: the assay of investigated compounds was performed following two or three transitions per compound, the first one for quantitative aim and the second and/or the third one for confirmation. In this study, various seven radiopaque material which are used widespread in clinic materials were quantified for effect of interference.

2.3. Preparation of Samples and Statistic:

100 microliters (µL) of control solution was transferred to a centrifuge tube. 200 µL of internal standard and 10 µL of distilled water were added and mixed for 5 seconds in vortex. The resulting mixture was centrifuged at 10,000 rpm for 10 minutes. The supernatant was transferred into a vial and measured in Shimadzu 8045 LC-MS/MS device. For the interference study, 10 µL of radiopaque agent was added into the plasma and measurement was made after mixing with a vortex. Each measurement was repeated 3 times and area values as well as concentration values were calculated by means of the Shimadzu Software. This procedure was repeated individually for each of the 7 different radiopaque

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agents. Area and concentration values for each of the three measurements were calculated with mean values for each mixture. Results derived by adding 10 µL of distilled water into the control material to exclude interference due to volume expansion were considered as target values. Radiopaque agents were coded from RM1 to RM7 instead of their commercial names due to copyright issues of trading companies. No ethics committee approval was required since no blood or tissue samples of human or animal origin was used in this study. (V2: Concentration of immunosuppressant with radiopaque material, V1: Concentration of immunosuppressant with distilled water)

$$\text{bias}(\%) = \frac{V2 - V1}{V1} x100$$

3. Results

Bias values were calculated by interference studies for each of the 7 different radiopaque agents. A false positive deviation of 153.72% was observed in everolimus level after the administration of RM3, which contains iohexol. Target level for everolimus was measured as 31.91 ng/mL while it should have been 12.58 ng/mL. Radiopaque agents coded RM1, RM5 and RM6 led to false negative results in everolimus levels by -10.06%, -17.51%, -4.04%, while RM2, RM4 and RM7 resulted in false positive results by 32.49%, 25.89%, and 4.6%, respectively. A false positive result of 171.41% was measured in sirolimus level after the administration of RM3, which contains iohexol. A false positivity of 114.01% was measured due to the radiopaque agent RM4, which contains gadopentetate dimeglumine (table1). False positive results ranging from 2.11% to 87.44% were observed after the administration of RM1, RM2, RM5, RM6 and RM7. False negativity ranging between 19.77%-29.28% were observed for tacrolimus levels and between 31.57%-44.45% for Cyclosporine A levels after the administration of 7 different radiopaque agents (figure1).

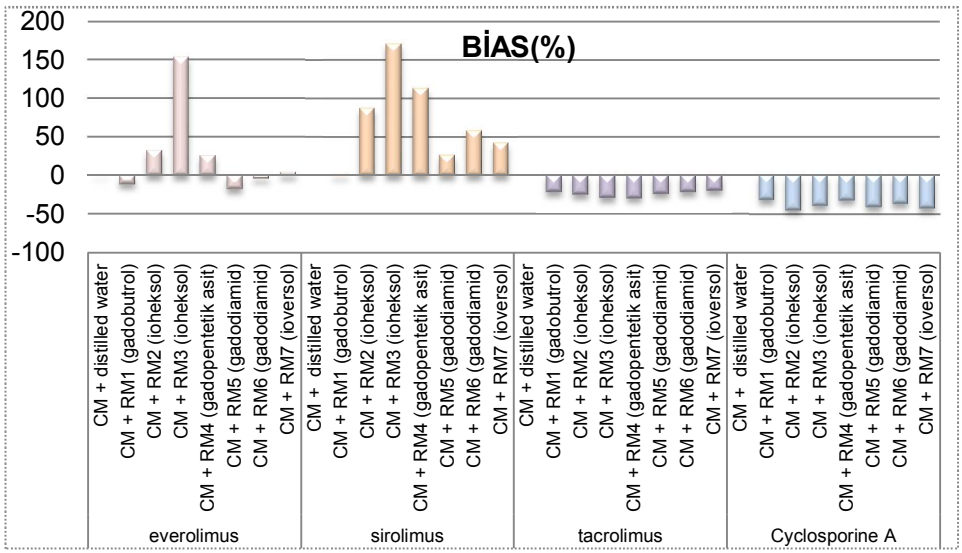

Figure 1. Graphic of percentage of deviation (bias) from target values

Table 1. Immunosuppressant concentrations and percentage of deviation (bias) from target values after radiopaque administration. (CM: Control Material, RM1-7: Radiopaque material)

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Drug		Retention Time (sec)	Concentration (ng/mL)	Amount of deviation (ng/mL)	BIAS (%)	Active Substance
Everolimus	CM + Distilled water	3.95	12.58	-	-	-
	CM + RM1	3.94	11.20	-1.38	-10.96	Gadobutrol, 604.72 mg/mL
	CM + RM2	3.94	16.67	4.09	32.49	Iohexol, 755 mg/mL
	CM + RM3	3.95	31.91	19.34	153.72	Iohexol, 755 mg/mL
	CM + RM4	3.95	15.84	3.26	25.89	Gadopentetate dimeglumine salt, 469.01 mg/mL
	CM + RM5	3.95	10.38	-2.20	-17.51	Gadodiamide, 287 mg/mL
	CM + RM6	3.96	12.07	-0.51	-4.04	Gadodiamide, 287 mg/mL
	CM + RM7	3.95	13.16	0.58	4.60	Ioversol, 741 mg/mL
Sirolimus	CM + Distilled water	3.86	13.78	-	-	-
	CM + RM1	3.90	14.07	0.29	2.11	Gadobutrol, 604.72 mg/mL
	CM + RM2	3.89	25.84	12.05	87.44	Iohexol, 755 mg/mL
	CM + RM3	3.91	37.41	23.63	171.41	Iohexol, 755 mg/mL
	CM + RM4	3.89	29.50	15.71	114.01	Gadopentetate dimeglumine salt, 469.01 mg/mL
	CM + RM5	3.88	17.50	3.72	26.96	Gadodiamide, 287 mg/mL
	CM + RM6	3.90	21.92	8.14	59.02	Gadodiamide, 287 mg/mL
	CM + RM7	3.89	19.68	5.90	42.81	Ioversol, 741 mg/mL
Tacrolimus	CM + Distilled water	3.29	12.22	-	-	-
	CM + RM1	3.34	9.57	-2.65	-21.68	Gadobutrol, 604.72 mg/mL
	CM + RM2	3.30	9.19	-3.02	-24.73	Iohexol, 755 mg/mL
	CM + RM3	3.30	8.68	-3.54	-28.96	Iohexol, 755 mg/mL
	CM + RM4	3.33	8.64	-3.58	-29.28	Gadopentetate dimeglumine salt, 469.01 mg/mL
	CM + RM5	3.34	9.34	-2.88	-23.57	Gadodiamide, 287 mg/mL
	CM + RM6	3.32	9.58	-2.64	-21.60	Gadodiamide, 287 mg/mL
	CM + RM7	3.29	9.80	-2.42	-19.77	Ioversol, 741 mg/mL
Cyclosporine A	CM + Distilled water	4.14	204.65			
	CM + RM1	4.15	140.05	-64.61	-31.57	Gadobutrol, 604.72 mg/mL
	CM + RM2	4.14	113.69	-90.96	-44.45	Iohexol, 755 mg/mL
	CM + RM3	4.15	125.82	-78.83	-38.52	Iohexol, 755 mg/mL
	CM + RM4	4.15	139.62	-65.04	-31.78	Gadopentetate dimeglumine salt, 469.01 mg/mL
	CM + RM5	4.14	122.38	-82.28	-40.20	Gadodiamide, 287 mg/mL
	CM + RM6	4.15	130.86	-73.79	-36.06	Gadodiamide, 287 mg/mL
	CM + RM7	4.15	117.73	-86.93	-42.48	Ioversol, 741 mg/mL

4. Discussion

Radiopaque agents are paramagnetic intravenous diagnostic drugs used in imaging techniques. Commonly used active substances in routine practice include iohexol, gadobutrol, gadopentetate dimeglumine salt, gadodiamide and ioversol . These agents may interfere with test results of the patient when measurements are performed in blood samples collected after imaging techniques [12,13]. The degree of this interference can change according to the elimination time of these drugs. In particular, the impact on the results with tacrolimus, sirolimus, cyclosporine A and everolimus,

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which are used for the immunosuppression of liver transplant patients are important for prognosis of patient [14]. Various studies have been carried out on incorrect measurement of immunosuppressants by immunoassay methods.

Elevated blood cyclosporine levels due to the presence of endogenous antibodies were reported by Soldin et al. in their ACMIA immunoassay measurements performed using the Dimension RXL analyzer. De Jonge et al. reported an incorrect cyclosporine level of 492 ng/mL in a 77 year-old patient. However, any cyclosporine molecules could not detect in this patient's blood by measurement of LC-MS. [15]. Sirolimus is also exposed to the interference by metabolites during immunoassay measurements. Morris et al. found a bias of 49.2% with MEIA (microparticle enzyme immunoassay) method compared to the measurements with LC MS/MS [16]. Schmidt et al. evaluated the sirolimus analysis by CMIA (carbonylmetallo immunoassay) method and found cross-reaction with sirolimus metabolites. In another study that compared CMIA and LC-MS/MS, deviations of 14% to 39% were observed between mean values. Higher results were found using the CMIA method compared to LC-MS/MS [17].

In a study that used 90 samples for everolimus levels with QMS (Quantitative Microsphere System) immunoassay method, everolimus values determined using the QMS everolimus test were found to be approximately 11% higher than those obtained by the LC-MS/MS method [18]. In a study by Hoffer et al. with 169 patient samples, mean everolimus concentration produced by the QMS everolimus test was found to be 31.2% higher than that determined by LC-MS/MS. [19]. Sallustio et al. observed a deviation of 30% between everolimus values measured by FPIA and LC-MS/MS methods [20].

Although drug metabolites are the main cause of interference in tacrolimus measurements, incorrect tacrolimus concentrations were reported with low hematocrit values by the MEIA (microparticle enzyme-linked immunoassay) method on AxSYM instrument [21]. Westley et al. found a bias of 33.1% and 20.1% when LC-MS/MS method was compared with CEDIA and MEIA methods, respectively, in renal transplant patients [22]. Bazin et al. evaluated tacrolimus test on the CMIA (Chemiluminescent Microparticle Immunoassay) method and observed an average bias of 20% compared to the values found using LC-MS/MS [23]. ACMIA tacrolimus test is affected by rheumatoid factors and endogenous heterophilic antibodies. Altinier et al. described an interference by heterophilic antibodies on ACMIA tacrolimus method. Therapeutic levels of tacrolimus were found in a patient resulting from the presence of heterophilic antibodies even after the treatment was discontinued [24].

Despite the fact that superiority of the LC-MS/MS method compared to immunoassay has been demonstrated in several studies, no study has been performed to investigate the impact of radiopaque agents used for organ function imaging in transplant patients on immunosuppressant levels. Analyte results may change by the matrix effect observed as the change in ionization activity in LC-MS/MS measurement in the presence of combustible substances [25]. Although it appears reliable for some clinicians to use this reference method in certain vital tests, it should be kept in mind that false results may occur due to interferences during these measurements. In this interference study performed with the addition of 7 different commercial radiopaque agents, a significant influence was found on the concentrations of tacrolimus, everolimus, sirolimus and cyclosporine A. All of the radiopaque agents included in the present study led to false negative results in tacrolimus and cyclosporine A levels at a rate of 19.77% to 44.45%. False negativity may lead the clinicians to increase drug dose. The smallest deviations were seen in everolimus levels with the administration of RM6 (gadodiamide) and in sirolimus levels with RM1 (gadobutrol) at rates of -4.04% and 2.11%, respectively. RM3 (iohexol) resulted in false positivity of 153.72% and 171.41% in everolimus and sirolimus levels. Incorrectly high measurements of immunosuppressant levels may lead to using insufficient drug doses and increased risk of organ rejection. RM2 and RM3 contain iohexol, RM5 and RM6 contain gadodiamide. Different rates of deviation from target levels despite the same active ingredients in commercial products is thought to be caused by different excipients that constitute the polar and apolar structure of these products. This is supported by the study of Bonfiglio et al. reporting that the chemical nature of a component had a significant effect on the degree of the matrix effect. A study including four compounds of different polarities under the same mass

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spectrophotometric conditions showed that the most polar compound had the highest rate of ion suppression and the least polar compound was less influenced by ion suppression [26]. King et al. showed in a number of experiments that matrix effect is a consequence of the competition between nonvolatile matrix components between analytical ions during the shift to ionization phase [27]. In this study, the competition of molecules differed according to the diversity of radiopaque molecules. The formation efficacy of analyte ions depends on the matrix intensity that enter the electrospray ion source. Some studies have demonstrated that signal suppression is complicated in the manifestation of the matrix effect and involves several many factors. Gas phase proton transfer reactions and the competition at high viscosity are the major factors in the formation of the matrix effect [28].

5. Conclusions

Although LC-MS/MS is the reference method that provides high specificity, excellent sensitivity and precision for measurements of immunosuppressant drugs, factors of matrix origin should be carefully evaluated. It has been experimentally demonstrated by this study that an interference may occur in blood immunosuppressant levels due to radiopaque agents. False test results due to radiopaque agents may lead to incorrect drug dosing. Choice of radiopaque agents with minimal measurement errors is important to reduce the risk of interference. However, the least risky method is to obtain samples for drug level measurements before contrast-enhanced imaging. Clinicians should interrogate administration of radiopaque agents and the time of sampling in the event that suspicious results are obtained during the measurement of immunosuppressants.

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