

Selective Testosterone Analysis in Human Serum by LC-FAIMS-MS/MS

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Key Words

- TSQ Quantum Ultra
- Clinical analysis
- High-throughput
- Selectivity

Overview

Current high throughput clinical assays utilizing triple quadrupole mass spectrometry for the quantitation of testosterone can be further enhanced through the use of FAIMS (high-Field Asymmetric waveform Ion Mobility Spectrometry) coupled to a TSQ Quantum triple quadrupole mass spectrometer. This application note describes how a FAIMS-enabled TSQ Quantum improves the performance of a testosterone assay.

Introduction

Testosterone is the androgenic hormone primarily responsible for normal growth and development of male reproductive organs. Although testosterone production declines naturally with age, testosterone production may be compromised by diseased or damaged organs.

Women biosynthesize very low levels of testosterone, which makes quantitation extremely difficult. Estrogen replacement therapy may further reduce testosterone production, resulting in additional complications in its quantitation. In addition, endogenous interferences may prevent accurate and precise testosterone measurement.

In this study, an LC-MS/MS method was used together with the selectivity offered by FAIMS to quantify testosterone in human serum. FAIMS acts to remove chemical background and endogenous interferences resulting in more accurate and precise determinations for clinical samples than LC-MS/MS alone.

Experimental Conditions

Chemicals and Reagents

Testosterone and testosterone- d_3 (internal standard-IS) were purchased from Sigma-Aldrich (St. Louis, MO). HPLC grade methanol and formic acid were acquired from Burdick and Jackson (Muskegon, MI). All chemicals were used as received.

Sample preparation: Stripped human serum (Golden West Biologicals, Temecula, CA) was fortified with testosterone at the following concentrations: 2.5, 5, 10, 25, 50, 100, 250, 500, 1000, and 2500 pg/mL. Internal standard in 5% formic acid was added to a final concentration of 500 pg/mL. No further sample preparation was required.

Sample analysis: LC-MS/MS analyses were performed on a Thermo Scientific Surveyor LC system. The method used mobile phases A (0.1% formic acid in water) and B (0.1% formic acid in methanol) at a flow rate of 0.5 mL/min. Serum samples (90 μ L) were injected onto an LC-MS/MS extraction column. The analyte was back-flushed to the 2.1 \times 50 mm, 3 μ , Thermo Scientific Hypersil GOLD analytical column. The entire LC effluent from the sample injections was directed to the Ion Max source on a Thermo Scientific TSQ Quantum Ultra.

Additional gas-phase separation prior to entry of ions into the mass spectrometer was achieved by including FAIMS in the analysis.

FAIMS Conditions

Dispersion voltage	-5000 V
Outer bias voltage	35 V
Compensation voltage	-12.5 V
Temperature (inner electrode)	60 °C
Temperature (outer electrode)	60 °C
FAIMS gas composition	50% He in N ₂
FAIMS gas flow rate	3.8 L/min

MS Conditions

Ionization mode and source	Positive APCI
Spray current	1.0 μ A
Vaporizer temperature	400 °C
Sheath gas	35
Transfer tube temperature	250 °C
Transfer tube offset	35 V
Tube lens offset	100 V
Collision energy	22 eV
Scan time	50 ms
Q1 Resolution	0.7 Da FWHM
Q3 Resolution	0.7 Da FWHM
Testosterone	m/z 289.2 \rightarrow m/z 97.1, 109.1
Testosterone- d_3	m/z 292.2 \rightarrow m/z 97.1, 109.1

Results and Discussion

LC-MS/MS is a highly selective technique for analyzing drugs from biological matrices. As shown in Figure 1, samples are loaded to the extraction column via an autosampler and LC pump combination. After a short washing time, the central valve is rotated 60° to the injection position, which allows a second pump to elute the analytes from the extraction column onto the analytical column and into the FAIMS-enabled mass spectrometer. In cases where background or co-eluting interferences appear, the limiting factor is selectivity.

FAIMS is a unique selectivity enhancing tool for LC-MS. With FAIMS, gas-phase ions are purified after LC analysis but before they are mass analyzed. The waveform shown in Figure 2 separates the gas-phase ions as they are transferred into the high vacuum region of the MS. The interference ions in orange are filtered out from the ion beam, while the analyte ions in blue pass into the MS.

The basic experiment that establishes FAIMS conditions is shown in Figure 3. While infusing a reference standard of testosterone into the mobile phase, the CV is scanned over a specific range. The black trace appears for the transition due to testosterone, and a maximum signal

for testosterone appears at CV -12.5 V. To ensure that mobile phase components with the same transition will be eliminated, stop the infusion of the reference standard and repeat the CV scan over the same specified range. The red trace that chemical background in the mobile phase emerges from FAIMS not at the CV for testosterone, but rather in a broad range between CV -15 and -25 V. For subsequent LC-FAIMS-MS/MS assays, the CV set to -12.5 V will exclude mobile phase contributions to the analysis of testosterone. Other endogenous components present in the human serum samples may also be excluded if their FAIMS behavior is different from that of testosterone.

Regression analysis based on a linear model with $1/[\text{concentration}]^2$ weighting was used. The average accuracy, as deviation from theoretical is less than 5% at all concentrations. The precision of the standards, as relative standard deviation (%RSD), is less than 17% at the lower limit of quantitation and less than 11% at all other concentrations.

Despite the excellent performance of LC at cleaning up samples, many interferences are still present from the matrix. Other extraction techniques might remove these

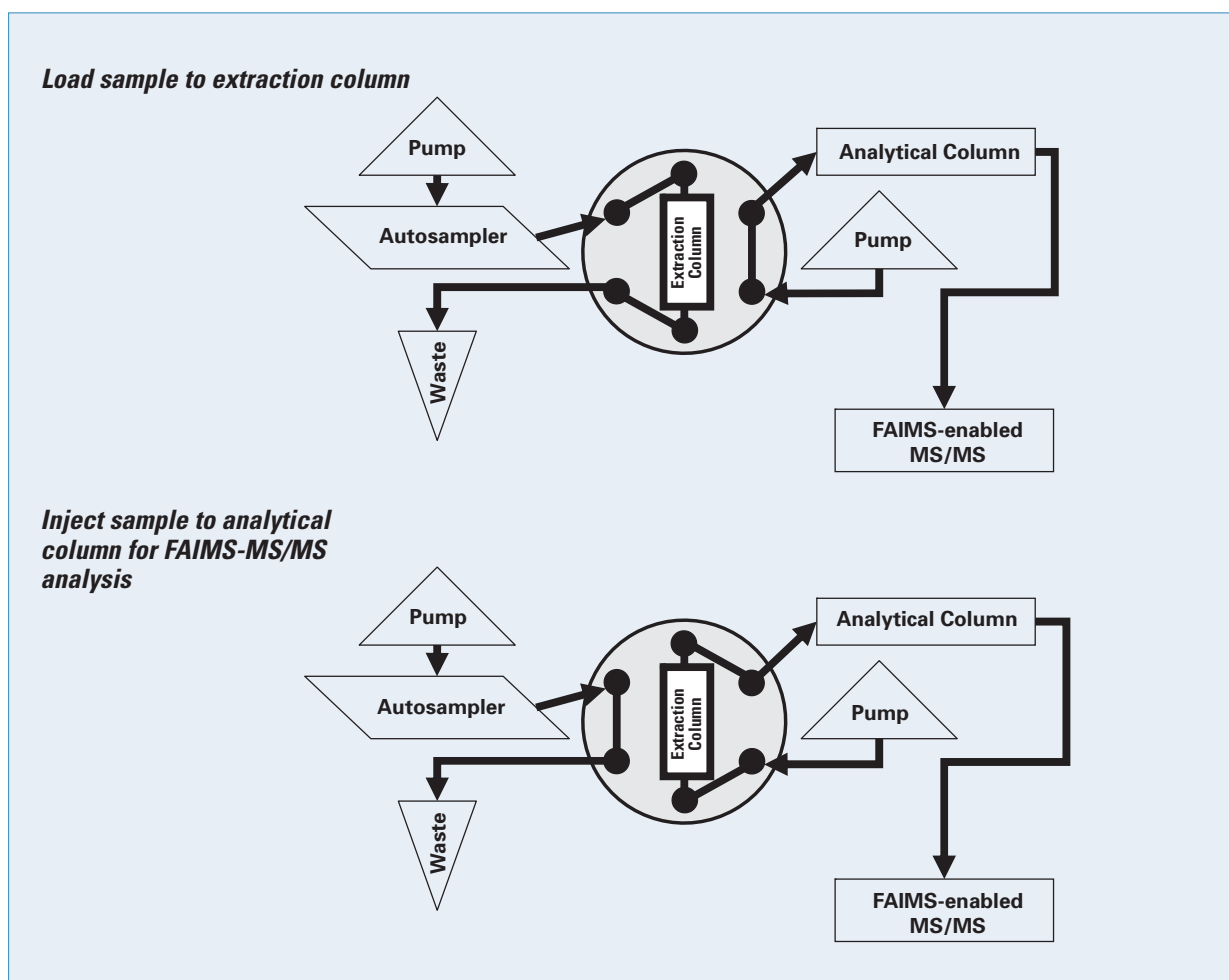


Figure 1: Schematic diagram of LC-FAIMS-MS/MS system

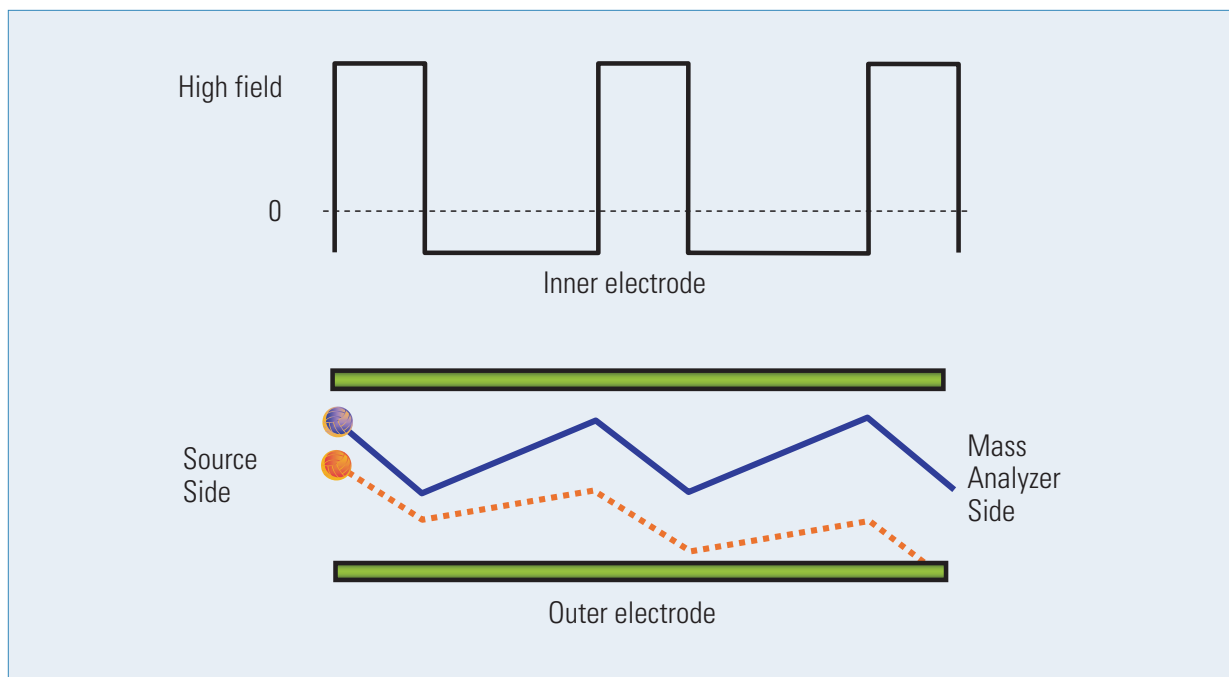


Figure 2: Compensation voltage set to transmit testosterone (blue ion, solid line). Testosterone and some of the interferences behave like the blue ion, while most of the interferences behave like the orange ion.

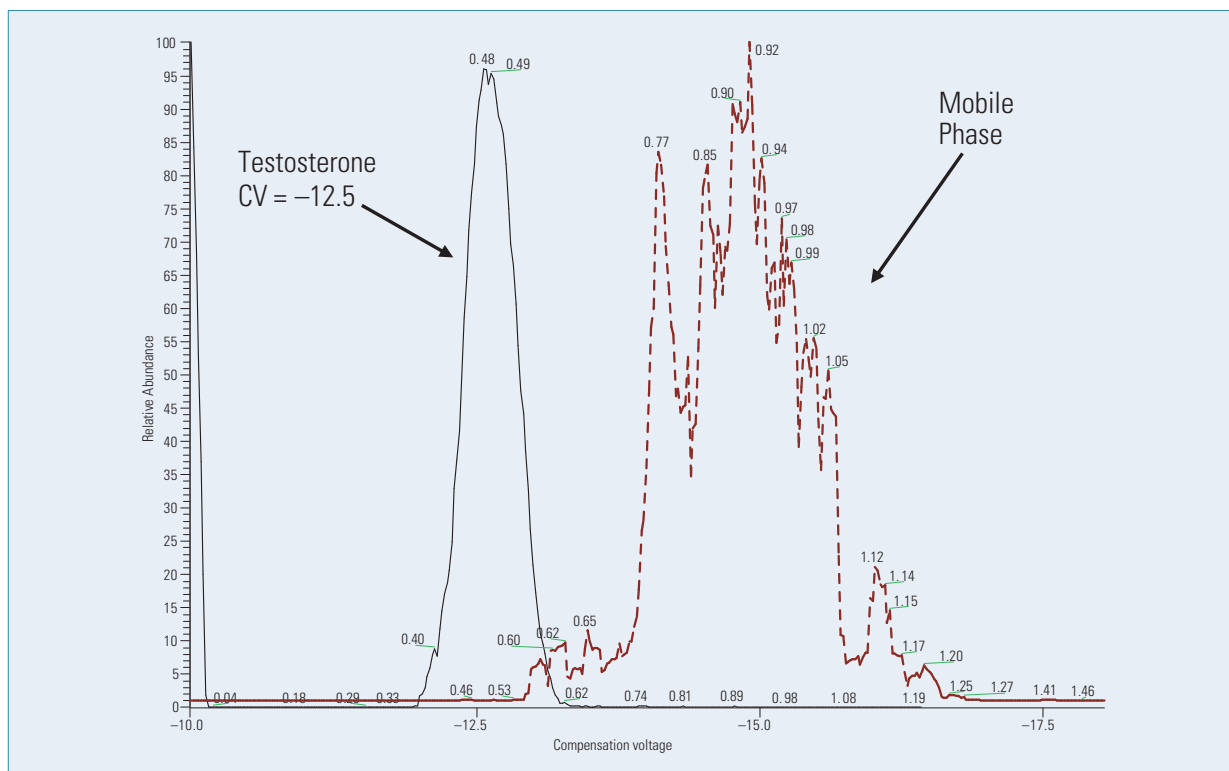


Figure 3: Compensation voltage scan for the infusion of testosterone reference standard in black. The red overlaid trace represents the CV scan from mobile phase alone.

interferences, but much time can be spent changing extraction selectivity. A representative LC-MS/MS chromatogram for testosterone in a human serum clinical sample is shown in the upper trace of Figure 4. Multiple interferences prevent accurate integration of the analyte. Note that the peak(s) at retention time 2.7 are not due to testosterone but rather multiple interferences. If this were the true concentration of testosterone the patient would not be able to survive having such a high endogenous level. The lower trace of Figure 4 shows the IS in human serum. Multiple interferences and an elevated baseline due to chemical background make peak integration difficult.

Figure 5 shows the same sample analyzed with FAIMS included in the method. LC-FAIMS-MS/MS of testosterone in the upper trace shows that many of the interferences of Figure 4 are removed. Correct peak integration for testosterone is now possible. The lower trace for the IS shows that the chemical background and interferences were eliminated. The use of FAIMS together with LC and tandem MS has improved the selectivity of the assay, resulting in a very accurate and precise method. The lower level of quantitation was improved four-fold more than the LC-MS/MS method.

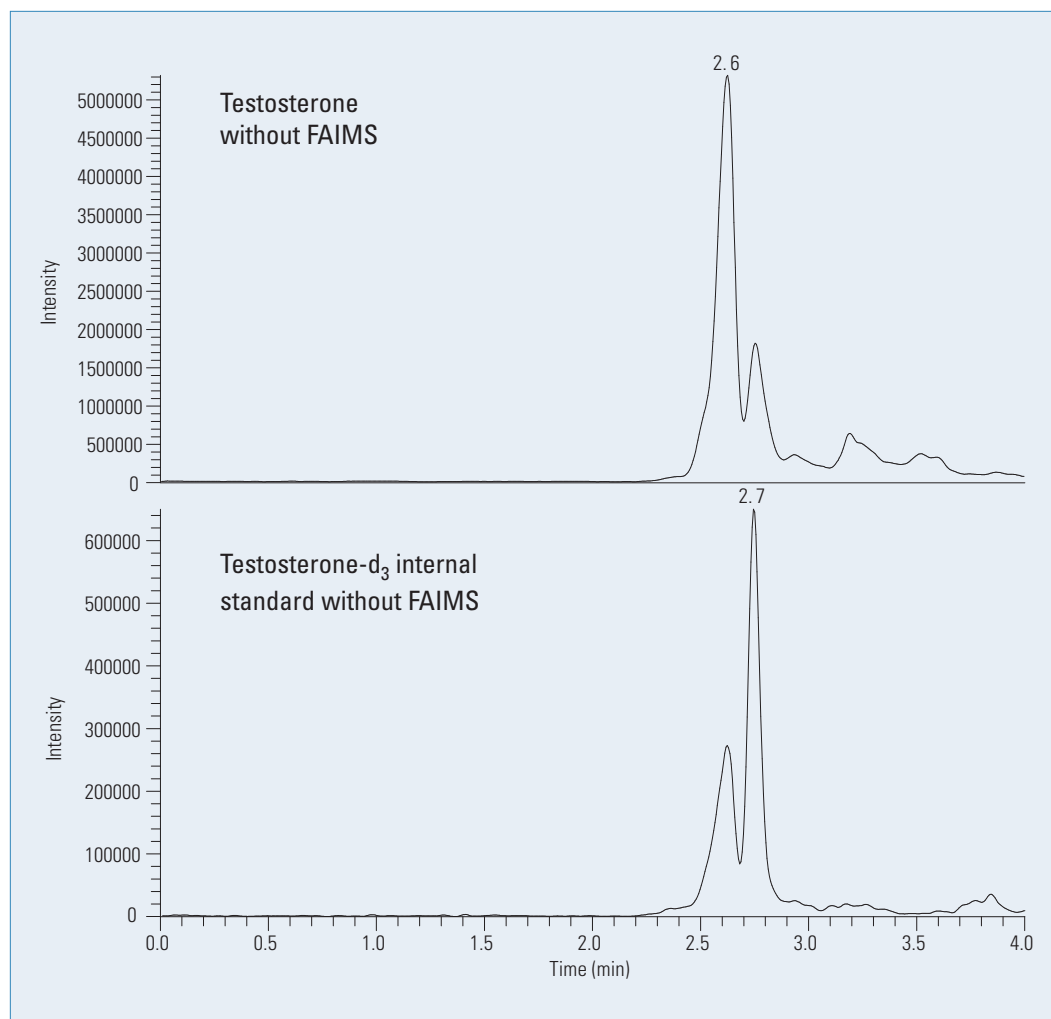


Figure 4: Representative LC-MS/MS chromatogram for testosterone in a human serum clinical sample. The upper trace is testosterone (retention time 2.7 min), the lower trace is IS (testosterone- d_3).

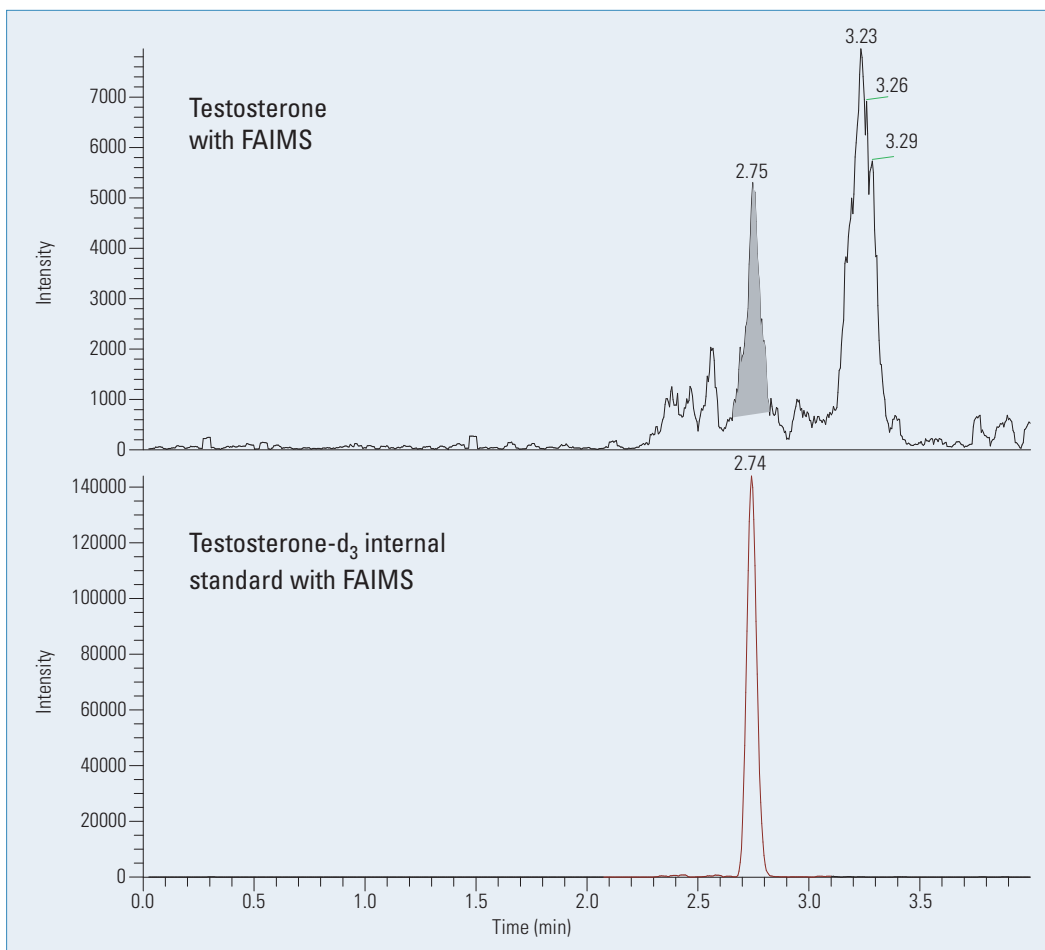


Figure 5: Using FAIMS. Representative LC-FAIMS-MS/MS chromatogram for testosterone and the lower trace is for the internal standard. Note that the absolute signal from FAIMS analysis is lower than without FAIMS because of the removal of interferences.

Conclusions

A FAIMS-equipped TSQ Quantum Ultra™ triple quadrupole mass spectrometer provides more accurate results by eliminating the chemical noise that arises from mobile phase and sample matrix. In cases where interferences prevent accurate and precise determination, FAIMS removes interferences. The resulting chromatograms accurately represent the concentration of testosterone in the samples. The LLOQ was improved four-fold compared to the LC-MS/MS method.

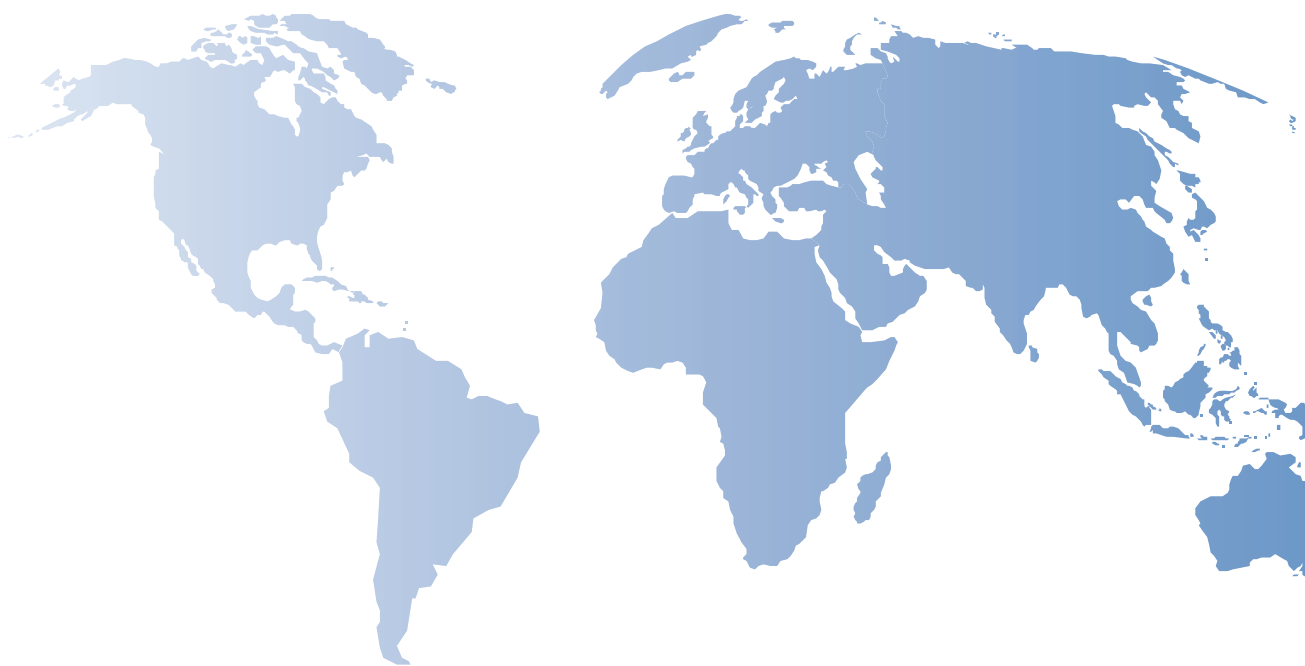
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