Application Note
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Using Heteroatoms as "Natural Labels" in the Quantitative Analysis of Active Pharmaceutical Ingredients by HPLC-ICP-MS

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Verified for Agilent
8900 ICP-QQQ

Introduction

LC-MS/MS is widely used in every stage of drug development, from discovery and characterization through manufacturing and quality control of the final pharmaceutical product. Due to its excellent selectivity for complex molecules, LC-MS/MS is an effective tool for both non-target qualitative analysis and quantitative analysis of targeted compounds. However, the sensitivity of LC-MS/MS varies significantly depending on the molecular structure of each compound, and can be affected by other coexisting substances in the sample. This limitation means that LC-MS/MS is often unable to provide accurate quantitative analysis without a compound-specific calibration standard. Inductively Coupled Plasma Mass Spectrometry (ICP-MS) is a fast multi-element technique capable of accurate elemental quantification at trace and ultra-trace levels. While more widely adopted in inorganic, "metals" laboratories, ICP-MS is also used in the field of pharmaceutical analysis when a heteroatom-containing drug is the focus of interest (*1*, *2*).

ICP-MS employs a high temperature plasma ion source that decomposes the sample to the atomic level. The high temperature plasma is much less prone to matrix-dependent signal suppression, and the elemental response is essentially independent of the compound structure, unlike LC-MS/MS. Because the sample compounds are decomposed in the plasma, ICP-MS cannot provide information on molecular structure, but its ability to provide accurate quantification based on the elemental response of heteroatoms contained in the compounds of interest means that ICP-MS is now being more widely applied for quantitative analysis in biological research (*3-6*).

ICP-MS can detect molecules/proteins indirectly by using a heteroatom contained within the targeted molecule/protein as an analyte "tag". The heteroatom may be present naturally, as in the sulfur-containing amino acids cysteine and methionine which may be incorporated into proteins. Alternatively, a metal "tag", typically a rare earth element (REE), may be added to the target molecule via chemical reaction (*7*) or immunoreaction (*8*). By measuring the signal of the REE, the concentration of the targeted molecule or protein can be determined based on the known element-to-molecule stoichiometry (the REE to molecule ratio). Adding an elemental tag has two merits. First, a tag element may be selected in order to achieve the lowest detection limit, i.e. an element that is easily ionized and free from spectral interferences. Gold (Au) and REEs are often used as they are rarely found in biological matrices. The second advantage is that the reaction process used to add the metal tag may itself provide excellent compound selectivity that the ICP-MS measurement doesn't have inherently. When immunoreaction is used, for example, it is possible to selectively tag only the molecule (small molecule or protein) of interest and therefore detect only the targeted protein. Elemental tagging with analysis by ICP-MS promises to be a useful technique for the detection and quantification of organic compounds, with good sensitivity and selectivity. When a targeted molecule naturally contains a heteroatom, the atom can be used to indirectly quantify the molecule's concentration by ICP-MS. For example a large number of Active Pharmaceutical Ingredients (API) contain sulfur (S), phosphorus (P), or halogens. In this case, no derivatization with an elemental tag is required, so absolute quantification is possible without any concern for post-derivatization recovery. Unfortunately, S, P and the halogens have a high first ionization potential so they are poorly ionized in the ICP-MS plasma, leading to low sensitivity.

S, P, and chlorine (Cl) are also difficult to measure by conventional quadrupole ICP-MS (ICP-QMS) due to intense spectral interferences. As a result, accurate analysis of S, P, and the halogens at the analytical ranges that are relevant to pharmaceutical molecules is nearly impossible to achieve by ICP-QMS. The alternative technique of Triple Quadrupole ICP-MS (ICP-QQQ), operating in MS/MS reaction cell mode, can be applied to resolve these spectral interferences, allowing the quantification of S, P, and Cl at far lower levels than was previously possible by ICP-QMS.

In this study, we investigated the measurement of molecules/proteins that contain the heteroatoms S, P, or Cl, the analysis of which is greatly improved with ICP-QQQ. Five APIs and a monoclonal antibody (mAb) were analyzed using ICP-QQQ. The targeted compounds included small (m = 250-320 Da) and large (m = 146 kDa for the mAb) molecules.

Experimental

Instrumentation

An Agilent 1260 Infinity Bio-inert HPLC system with quaternary pump (G5611A) and autosampler (G5667A) was interfaced to an Agilent 8800* Triple Quadrupole ICP-MS (G3663A, #100). An HPLC flow rate of 0.4 mL/min and an injection volume of 20 µL were applied throughout the study. Two types of columns were used: an Agilent ZORBAX plus C18, 2.1 x 100 mm, 3.5 um (Agilent # 959793-902) was used for the analysis of the small molecules, and an Agilent Bio SEC-3 300 Å, 4.6 x 150 mm, 3 um (Agilent # 5190-2514) was employed for the mAb analysis. Identification of each targeted molecule was based on its retention time. HPLC mobile phase compositions, and separation conditions for each method are given in the respective results and discussion sections.

The HPLC column exit was connected to one of the ports of a mixing-T used for on-line Internal Standard (ISTD) addition. ISTD solution was delivered via the ICP-MS on-board peristaltic pump and was mixed with the column eluate at the T-connector. 50 µg/L Co and Y were introduced as ISTDs for tuning purposes and the ISTDs were also used to monitor possible instrumental drift. However, no drift-correction was necessary as the signal was stable throughout the experiment. During the analysis of the Cl–containing drug, 20% NH_3 (aq) was introduced via the ISTD line to improve washout.

The Agilent 8800 instrument has been superseded by the 8900. This analysis could be performed on either instrument.

All ICP-MS measurements were carried out using the Agilent ICP-QQQ fitted with an x-lens, standard Peltier-cooled double-pass Scott-type spray chamber, glass concentric nebulizer, and platinum interface cones. A standard one-piece quartz torch with 2.5 mm internal diameter (ID) injector was used for the mAb analysis, and a 1.5 mm injector torch was used for analysis of the organic mobile phases used for the other samples. A blend of 20% oxygen in argon was introduced via the optional gas line to prevent carbon build-up on the interface cones during the introduction of the organic solvent mobile phases.

Optimization

The ICP-QQQ mass-shift method for S and P measurement (for analysis of the S-containing and P-containing APIs and the mAb) used O_2 cell gas, while the method for Cl measurement (for the Cl-containing API) used H_2 cell gas. Instrument optimization was performed based on the signal of the ISTD elements while introducing the respective mobile phase. The optimized tuning parameters are summarized in Table 1.

Table 1: Tuning parameters of the Agilent ICP-QQQ.

Parameter	Units	mAb	Sulfonamide	Zoledronic acid	Clonidine
RF power	W	1600			
Sampling depth	mm	8.0			
Carrier gas flow rate	L/min	0.9	0.6		
Makeup gas flow rate	L/min	0.25	0.0		
Option gas flow rate (20% O_2)	L/min	0.0	0.2		0.0
Octopole bias	V	-4.0			
KED	V	-8.0			
Cell gas flow rate	mL/min	O_2 = 0.3			H_2 = 3.0
Heteroatom		Sulfur		Phosphorus	Chlorine
Mass pair (Q1, Q2)		(32, 48)		(31, 47)	(35, 37)

Reagents

HPLC grade methanol was purchased from Kanto Chemicals (Tokyo, Japan). Acetonitrile, disodium hydrogen phosphate, sodium dihydrogen phosphate, formic acid, tetra-butyl-ammonium bromide, and acetic acid were purchased from Wako Pure Chemicals (Osaka, Japan). Sulfamethizole, sulfamethazine, sulfamethoxazole, zoledronic acid hydrate, and clonidine hydrochloride were purchased from Sigma Aldrich (St Louis, MO, US). Semiconductor grade NH_3(aq) was purchased from Tama Chemicals (Kanagawa, Japan). A monoclonal antibody (IgG2a) was obtained from Agilent Technologies (Agilent #200473).

Results and discussion

MS/MS reaction cell method to measure S, P, and Cl

APIs containing S, P, or Cl were measured using MS/MS mode on the Agilent ICP-QQQ. As mentioned, the measurement of these elements at low levels is challenging for ICP-QMS due to their low ionization and the fact that they suffer from intense, hard to resolve, polyatomic interferences. The Agilent ICP-QQQ with MS/MS capability uses mass-shift (*9*) to remove the spectral interferences on each element. In mass-shift mode, the target analyte ion is converted into a reaction product-ion by an ion-molecule reaction with the cell gas in the Octopole Reaction System (ORS) cell. The reaction product ion is shifted from its original analyte ion mass (e.g. by +16 amu in the case of O-atom addition reaction) and can therefore be detected free from the original interference. Uniquely, MS/MS mode eliminates the possibility of other existing or cell-formed ions overlapping the analyte at its new product ion mass, as the first quadrupole (Q1) specifically selects only the mass of the analyte precursor ion, so all other ions are rejected and cannot enter the ORS. MS/MS mode on ICP-QQQ therefore ensures that consistent results can be obtained when using reactive cell gases with variable sample matrices, which is a major benefit of the Agilent ICP-QQQ compared to reaction mode on ICP-QMS.

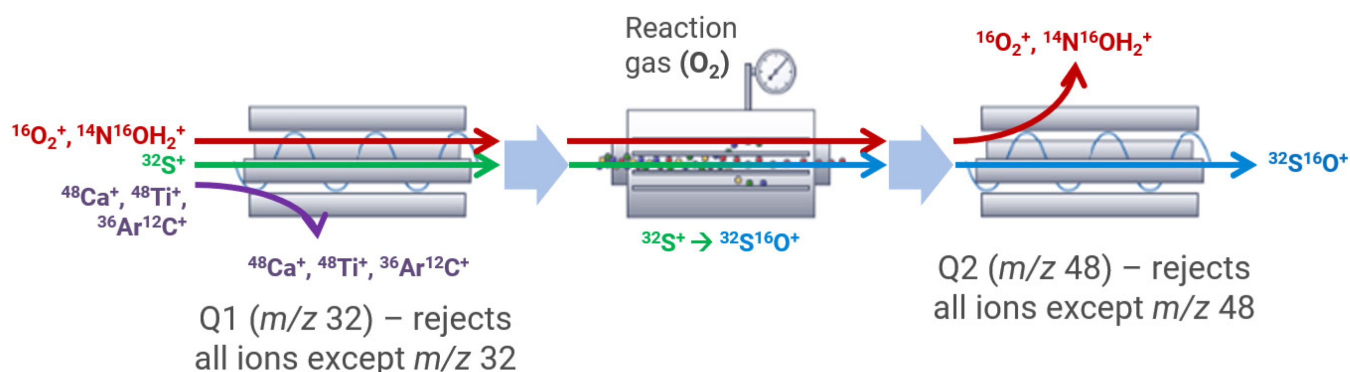


Figure 1. ICP-QQQ MS/MS mass-shift mode for the determination of ^{32}S as $^{32}S^{16}O^+$ using O_2 cell gas

Figure 1 illustrates how S is measured using MS/MS mass-shift with O_2 as the cell gas. In mass-shift mode, a reaction gas is chosen which selectively reacts with the analyte and not the interferences (the reverse applies in the case of on-mass measurement). For S determination using O_2 cell gas, the first quadrupole (Q1) is set to the mass of the target analyte precursor ion, in this case S^+ at *m/z* 32. In MS/MS mode, Q1 operates as a true mass filter (with 1 amu mass resolution), so only the analyte and any on-mass interfering polyatomic ions can enter the ORS cell and react with the O_2 cell gas. The second quadrupole, Q2, is set to the mass of the reaction product (oxide) ion of the element/isotope of interest (in this case *m/z* 48 for $^{32}S^{16}O^+$), so rejecting any interfering ions that did not react with the cell gas and therefore remained at *m/z* 32 (e.g. $^{16}O_2^+$ and $^{14}N^{16}OH_2^+$). P is measured (as $^{31}P^{16}O^+$ at *m/z* 47) using a similar mass-shift mode with O_2 cell gas. For Cl analysis, mass-shift mode with H_2 cell gas is used. Cl^+ (*m/z* 35) is converted to ClH_2^+ (*m/z* 37) via reaction with H_2 cell gas.

Sulfur-containing APIs

Sulfonamides are widely used as antimicrobial drugs as they inhibit synthesis of bacterial folate that bacteria require for DNA synthesis. Three sulfonamide APIs, sulfamethizole, sulfamethazine and sulfamethoxazole, were dissolved separately in methanol or methanol/water. Each sample was filtered, diluted with the LC mobile phase of 13% acetonitrile with 0.1% formic acid, and injected into the HPLC using an isocratic separation. The resulting overlaid chromatograms are shown in Figure 2. The method detection limit (MDL) for the compound sulfamethizole was calculated to be 23 nM (6.3 ppb as the compound and 1.5 ppb as S).

Biological therapeutics using monoclonal antibodies (mAb) is an emerging and potentially very powerful branch of immunotherapy. The therapy utilizes a mAb to attack specific antigens or cell processes such as cell division (*10*).

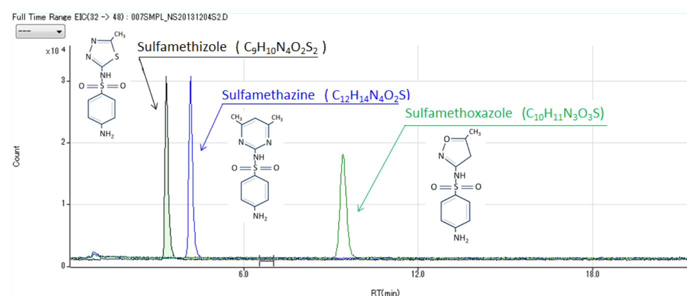


Figure 2. Overlaid chromatograms of three sulfur-containing APIs. The concentration of S in all 3 APIs injected is 100 ppb.

Antibodies are glycoproteins that contain about 1% sulfur and are therefore excellent targets for quantification via sulfur determination by ICP-QQQ. A mAb (IgG2A) obtained from Agilent was diluted with ultra pure water (UPW) and injected into the HPLC. An isocratic mobile phase of 50 mM phosphate buffer adjusted to pH 7.0 was used. Figure 3 shows the overlaid chromatograms obtained for two different concentrations of IgG2A. The MDL was calculated to be 14 nM (40 ng) as the compound.

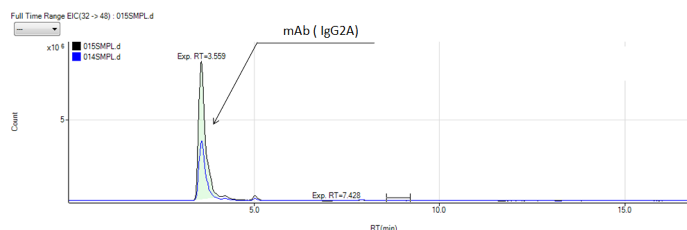


Figure 3. Overlaid chromatograms of 0.5 mg/mL and 1.0 mg/mL mAb (IgG2A) obtained by HPLC-ICP-QQQ.

Phosphorus-containing API

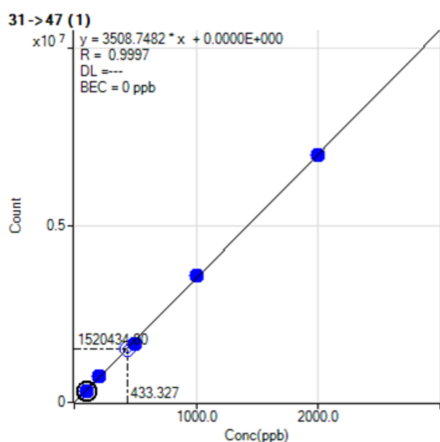
ZOMETA is a commercial drug that contains zoledronic acid hydrate ($C_5H_{10}N_2O_7P_2.H_2O$). A 5 mL vial of commercially supplied ZOMETA (containing 4.264 mg of the API) was prepared by diluting the drug 2000-fold with the LC mobile phase yielding a final API concentration of 426.4 µg/L. The isocratic mobile phase consisted of a 70:30 mixture of A: 6 mM tetra-butyl-ammonium bromide and 5 mM acetic acid adjusted to pH 6.5 with NH_3(aq), and B: 95% MeOH. A calibration curve was prepared using zoledronic acid hydrate standards, and the API in the sample was quantified based on the response for P compared to the external calibration. The calibration curve for P, measured as PO^+ at *m/z* 47, and the chromatogram of zoledronic acid hydrate measured in the ZOMETA sample are shown in Figure 4.

The concentration of the API in the sample was determined to be 433 ng/mL, which is a recovery of 102%. The MDL for the drug compound was calculated to be 25 nM (144 pg; 7.2 ppb as compound and 1.5 ppb as P).

Chlorine-containing API

Catapres is a commercial drug that contains clonidine hydrochloride ($C_9H_9Cl_2N_3.HCl$). A tablet of Catapres (containing 75 µg of the API) was dissolved in 50 mL water and sonicated for 60 minutes. The solution was then filtered and analyzed by HPLC-ICP-QQQ. A calibration curve was prepared by analyzing clonidine hydrochloride standards, and the API in the sample was quantified by external calibration. The isocratic HPLC method used a mobile phase consisting of 20% acetonitrile with 0.1% formic acid adjusted to pH 7.0 by NH_3(aq).

The calibration curve for Cl measured as $^{35}ClH_2^+$ at *m/z* 37, and the chromatogram of clonidine hydrochloride are presented in Figure 5. The concentration of the API in the sample was determined to be 1444 ppb, which is a recovery of 96%. The MDL of the drug compound was 146 nM (780 pg; 39 ppb as compound and 15 ppb as Cl).



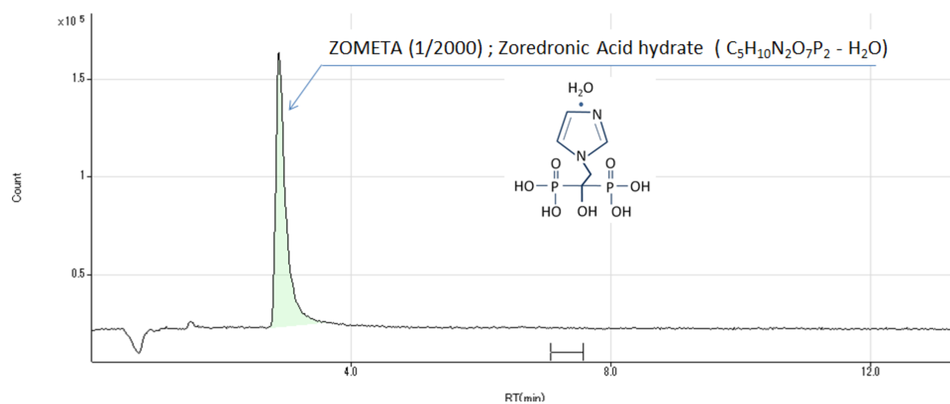
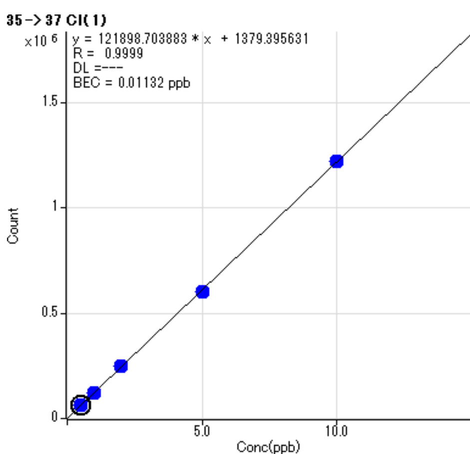


Figure 4. (Left) Calibration curve for P (as $^{31}P^{16}O^+$). (Right) Chromatogram of zoledronic acid hydrate ($C_5H_{10}N_2O_7P_2.H_2O$) in ZOMETA sample.



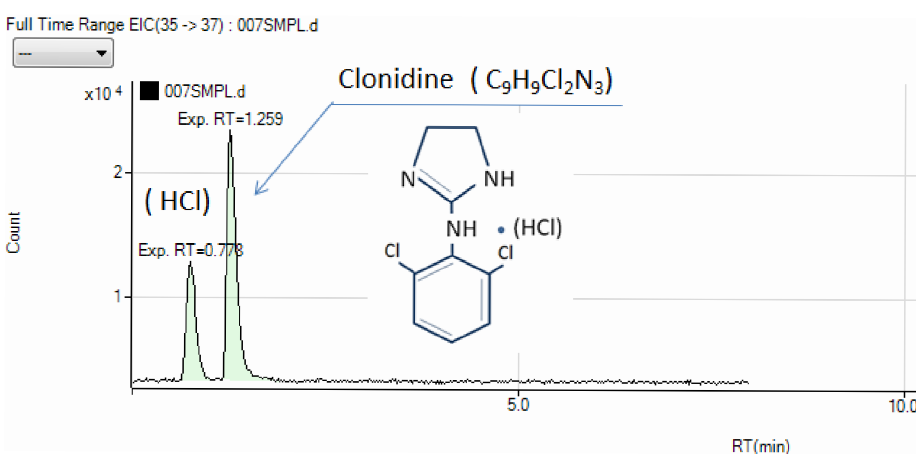


Figure 5. (Left) Calibration curve for Cl (as $^{35}ClH_2^+$). (Right) Chromatogram of clonidine hydrochloride ($C_9H_9Cl_2N_3.HCl$) in Catapres

Conclusion

The advanced capabilities of the Agilent 8800 ICP-QQQ operating in MS/MS mode has been successfully applied to the analysis of drug APIs and mAb, based on the measurement of the heteroatoms S, P, and Cl - an analysis that is normally carried out using molecular-MS techniques. These preliminary studies are presented here in order to demonstrate the potential use of HPLC-ICP-QQQ in drug development and post-manufacturing QA/QC control.

Future investigations are currently underway to explore the low detection capability of the ICP-QQQ. By using heteroatoms, ICP-QQQ promises to be a useful analytical tool in the bio-analytical and pharmaceutical industries, while also being applicable for conventional metal analysis, including the measurement of metal-tagged biomolecules.

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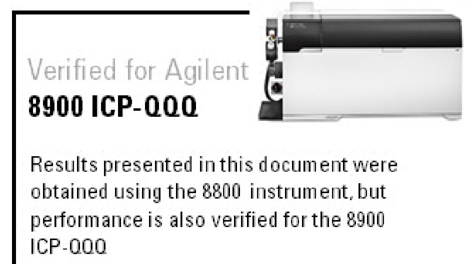

Results presented in this document were obtained using the 8800 instrument, but performance is also verified for the 8900 ICP-QQQ

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