Application Note

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Determination of Diclofenac and Its Related Compounds using RP-HPLC-ICP-QQQ

Compound structure-independent quantification of drugs

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Introduction

Quantitative drug metabolite profiling is an important application in the pharmaceutical industry. Researchers involved in drug development require an analytical technique with a response that is independent of compound structure. This compound-independent response enables accurate quantification of the drug and its metabolites, without requiring compound-specific calibration. Currently, radiolabeling techniques followed by HPLC separation and radiodetection are used for this application, but a simpler, quicker, and safer alternative approach is desirable.

The very high temperature plasma ion source and elemental ion-based measurement of ICP-MS enables compound structure-independent quantification, so individual standards for the metabolites of the (candidate) drug are not required. ICP-MS also links seamlessly with chromatography systems for speciation studies, for example HPLC.

HPLC-ICP-MS is used in a wide range of applications, including speciation studies of metals and metalloids, such as arsenic, mercury, selenium, chromium, and antimony [1]. However, many drugs contain nonmetal heteroatoms such as phosphorus, sulfur, chlorine, fluorine, or bromine, rather than metals and metalloids. The determination of these nonmetals is difficult by conventional single quadrupole ICP-MS, due to poor ionization, spectral overlaps, high backgrounds, or a combination of these factors. Except for F, these "difficult" elements can be measured accurately at low levels by triple quadrupole ICP-MS (ICP-QQQ) operating in MS/MS mode with a reactive cell gas. Reversed phase (RP) HPLC coupled to ICP-QQQ can introduce further analytical challenges due to the changing composition of the mobile phase gradient. In this study, it was necessary to compensate for the effect of gradient elution on the instrumental response during the RP-HPLC-ICP-QQQ analysis [2].

This note describes the quantitative determination of the drug diclofenac and its related compounds in human plasma. Diclofenac is a prescription non-steroidal anti-inflammatory drug (NSAID) that is used to alleviate mild to moderate pain, fever, and inflammation. Compounds were quantified based on measurement of the Cl heteroatom using RP-HPLC-ICP-QQQ.

Chlorine is not a typical analyte for ICP-MS, due to its poor ionization, high background signal, and the presence of intense spectral overlaps. The element's very high first ionization potential of 12.967 eV means that Cl atoms are only converted to positive ions (Cl^+) with an efficiency of about 0.13 % in an argon plasma operating at a nominal temperature of 7000 K. Chlorine is also a common contaminant in the laboratory, either from handling of sample containers, sample preparation equipment, or instrument hardware. Also, HCl acid is commonly used for stabilization of many elements, and chlorine tablets are often used as a biocidal treatment in deionized water systems, leading to high background. Finally, both isotopes of Cl (^{35}Cl and ^{37}Cl) suffer from polyatomic interference from polyatomic ions including O_2H^+, SH^+, and ArH^+.

Experimental

Samples

Diclofenac sodium (99.9% purity) and 4'-hydroxydiclofenac (99.0% purity) were bought from Sigma-Aldrich (St. Louis, MO, USA). Mixed working solutions containing diclofenac sodium and 4'-hydroxydiclofenac were used for method development, external standard calibration, and method validation. Full details are given in Reference 2. Diclofenac was synthetically degraded to generate degradation products covering a broad hydrophobicity range [2]. The synthetically degraded diclofenac samples were used as part of the mass balance study.

Human blood plasma was collected from healthy individuals, pooled, and stored at -20 °C until analysis. Sample preparation details are given in Reference 2.

Instrumentation

An 8800* Triple Quadrupole ICP-MS (ICP-QQQ) was used for all measurements; the instrument was fitted with a PFA nebulizer and platinum cones. The spray chamber was set to a temperature of -1 °C and a plasma torch with a 1.0 mm internal diameter injector was used. These changes helped ensure plasma stability with the high vapor pressure from the volatile organic solvent-based mobile phase (B). Oxygen (20% O_2 in Ar) was added to the carrier gas flow at 0.20 L/min to prevent the build-up of carbon on the interface.

To address the spectral overlaps on Cl, the major isotope ^{35}Cl (75.78% abundance) was measured by ICP-QQQ in MS/MS mode using a mass-shift method with H_2 cell gas. In this mode, ICP-QQQ avoids the interferences on ^{35}Cl by measuring the product ion $^{35}ClH_2^+$ at ***m/z*** 37 [3]. ICP-QQQ operating conditions and parameters are given in Table 1.

Table 1. ICP-QQQ operating conditions and acquisition parameters.

RF power (W)	1570
Ar carrier gas flow rate (L/min)	0.30
Optional gas (20% O_2 in Ar) mass flow controller setting	20% (0.2 L/min)
Spray chamber temp (°C)	-1
H_2 cell gas flow rate (mL/min)	3.5
Monitored transitions/masses, Q1 → Q2 (*m/z*)	35 (Cl^+) → 37 (ClH_2^+)
Data collection mode	TRA
Integration time (s)	0.4 for *m/z* 37

The ICP-QQQ was coupled to an Agilent 1260 Infinity HPLC System equipped with an Agilent 1260 Infinity Vacuum Degasser, an Agilent 1260 Infinity Binary Pump, an Agilent 1260 Infinity Autosampler, an Agilent 1290 Infinity Thermostatted Column Compartment, and an Agilent 1290 Infinity Series 2-position/10-port Microvalve. Column details and operating conditions are given in Table 2.

To compensate for the increased sensitivity for Cl caused by the changing level of acetonitrile during the gradient elution, a mathematical correction was applied to the Cl response (measured as $^{35}ClH_2^+$). The correction was based on the measured variation of the Cl response with increasing acetonitrile concentration, as shown in Figure 1.

The chromatographic peaks for the drug and metabolite compounds were identified by retention times (RT), and

* The 8800 ICP-MS has been superceded by the 8900 model.

each peak area was then integrated. The organic solvent concentration at the RT of each peak was calculated from the LC gradient program. The appropriate response factor for each peak was then determined from the organic solvent concentration and the response curve. Finally, the corrected Cl concentration of each peak was calculated based on the peak area and the corresponding response factor.

Table 2. HPLC operating conditions.

	Online preconcentration
Analytical column	Waters XBridge BEH C18 (4.6 x 150 mm; 3.5 µm)
Eluent A	0.1% (v/v) formic acid in MQ water
Eluent B	0.1% (v/v) formic acid in acetonitrile
Gradient	0–25 min: 70 → 0% A (30 → 100% B) 25–26 min: 0% A (100% B)
Flow rate (mL/min)	1.0
Sample temp (°C)	5
Column temp (°C)	22–23 (room temp)
Injection volume (µL)	50

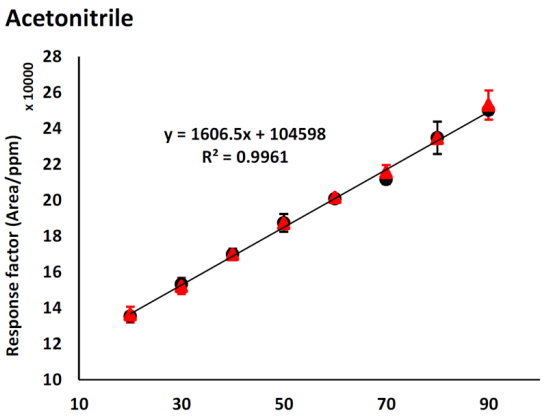


Figure 1. Measured response curve demonstrating the effect of the organic solvent (acetonitrile) content of the mobile phase on the Cl response of ICP-QQQ for both inorganic Cl and diclofenac-Cl (95% confidence intervals, n = 3). The response factor was found to be independent of the chemical form, as expected.

Method Development and Method Validation

Selectivity

Compound selectivity—the ability of a technique to distinguish an individual compound from other (often related) compounds—was confirmed by comparing the chromatograms shown in Figure 2. The chromatograms include (i) a blank, (ii) a mixture of 4′-hydroxydiclofenac and diclofenac, each at a concentration equivalent to 1 mg/L (ppm) Cl (iii) synthetically degraded diclofenac at a concentration equivalent to 10 mg/L Cl, and (iv) synthetically degraded diclofenac (at 10 mg/L Cl) spiked with 4′-hydroxydiclofenac (at 1 mg/L Cl).

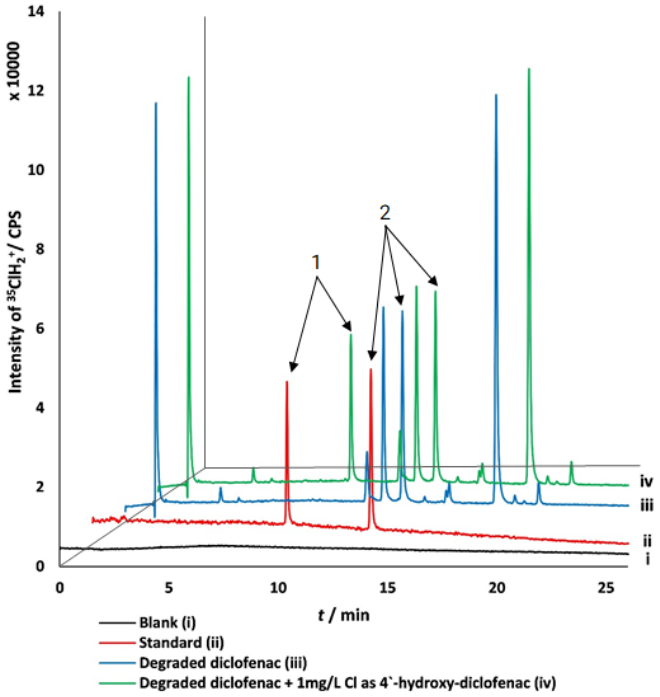


Figure 2. Chlorine chromatograms for (i) a blank, (ii) 4′-hydroxydiclofenac plus diclofenac, each at a concentration equivalent to 1 mg/L Cl, (iii) synthetically degraded diclofenac at 10 mg/L Cl, and (iv) synthetically degraded diclofenac at 10 mg/L Cl, spiked with 4′-hydroxydiclofenac at 1 mg/L Cl.
Peak 1: 4′-hydroxydiclofenac; peak 2: diclofenac; other peaks: degradation products with unknown chemical structure

Accuracy and precision

Accuracy and precision were investigated by spiking blank human plasma with 4′-hydroxydiclofenac and diclofenac at three concentration levels (three replicates at each level). The recovery was determined for both compounds. To assess the precision of the method, both intraday and interday precision were studied. As summarized in Table 3, excellent results were obtained with recoveries between 90-100% and RSDs below 4%.

Linearity and Limit of Quantitation

The linearity of the method was tested by injecting diclofenac standard solutions at concentrations equivalent to between 0.05 and 5.0 mg/L Cl. Excellent linearity was achieved with an $R^2 > 0.99$ and with the origin included in the 95% confidence interval of the intercept.

The limit of quantification (LOQ) was determined according to the signal-to-noise (S/N) method described in the ICH Q2(R1) guidelines (Part II, section 7.2). The LOQ for diclofenac was a compound concentration equivalent to 0.05 mg/L Cl.

Table 3. Accuracy and precision of the results for 4'-hydroxydiclofenac and diclofenac spiked into human plasma matrix.

Cl conc (mg/L)	Recovery (%)		Intraday precision (RSD%)		Interday precision (RSD%)	
	4'-hydroxy-diclofenac	Diclofenac	4'-hydroxy-diclofenac	Diclofenac	4'-hydroxy-diclofenac	Diclofenac
0.5	92.4	97.5	2.7	3.2	3.1	2.5
1.0	95.0	97.2	1.8	1.8	1.9	2.3
3.0	91.9	91.8	0.2	0.3	0.3	0.6

Table 4. Comparison of mass balance studies with and without human plasma sample matrix.

RT/min	v/v % of acetonitrile*	Cl conc without plasma (mg/L)	Cl conc with plasma (mg/L)	Relative difference (%)**
1.4	30.0	2.10	2.10	0.0
4.4	38.3	0.10	0.10	-2.3
11.1	57.1	0.43	0.44	3.3
11.8	59.2	1.29	1.30	1.5
12.7	61.6	1.25	1.27	1.0
14.7	67.2	0.07	0.07	-9.3
14.8	67.6	0.15	0.14	-5.6
17.0	73.6	2.89	2.94	1.5
17.8	76.0	0.05	0.06	12.3
18.9	79.0	0.15	0.16	0.2
Measured total Cl content (mg/L)		**8.49**	**8.57**	
True spike amount (mg/L)		**9.21**	**9.21**	
Recovery (%)		**92.2**	**93.1**	

**The eluent composition in which the compound with the indicated retention time is eluted.*
***Results in spiked plasma, relative to the values obtained without human plasma matrix.*

Mass balance study

The mass balance study was performed using a blank solution and a human plasma matrix. Each solution was spiked with synthetically degraded diclofenac at a level equivalent to a nominal total Cl concentration of 10 mg/L (the actual total spike amounts are shown in Table 4). The total Cl content of all the compounds in the spiked samples was measured and the concentration and recovery results are given in Table 4.

The recovery for the total Cl content was excellent, both in the absence and presence of the human plasma matrix (92 and 93 %, respectively). This matrix-independent response was further confirmed by comparing the results separately for each degradation product peak with and without the plasma matrix. The relative percent differences (RPDs) observed were mostly less than 5%. Slightly higher differences—up to 12% RPD—were observed for compounds that were present at levels close to the LOQ of 0.05 mg/L. It can be concluded that the human plasma matrix does not introduce any bias to the results obtained using this method.

Enhancing Sensitivity for Cl

Online sample preconcentration

To improve the sensitivity of the method and enable metabolite profiling of low-dose Cl-based drugs, a simple sample preconcentration procedure was used. The drug-related compounds present in human plasma were trapped on a trapping column (Waters XBridge BEH C18 4.6x20 mm; 3.5 µm) before analytical separation and ICP-QQQ detection. No additional sample pretreatment was required. The injection volume was increased to 1500 µL to load the preconcentration column. More details can be found in Reference 2. Using preconcentration, the LOQ for diclofenac was equivalent to 0.002 mg/L Cl – a 25-fold improvement. Human plasma blanks were spiked with 4'-hydroxydiclofenac and diclofenac at three concentration levels between 0.005 and 0.05 mg/L Cl (5 to 50 µg/L, ppb). Excellent recoveries between 94 and 98% were obtained for both compounds at all concentration levels, as shown in Table 5.

Table 5. Recoveries obtained for 4'-hydroxydiclofenac and diclofenac in the presence of human plasma matrix, when using a simple sample preconcentration procedure.

Cl conc (µg/L, ppb)	Recovery (%)	
	4'-hydroxydiclofenac	Diclofenac
5	95.7	96.7
30	97.8	95.7
50	97.4	93.9

Conclusions

A reversed phase HPLC-ICP-QQQ method has been successfully used for the compound-independent quantitative determination of diclofenac and its related compounds. Based on the measurement of the Cl heteroatom, the new HPLC-ICP-QQQ approach is quicker, simpler, and safer than the traditional radiolabeling HPLC technique.

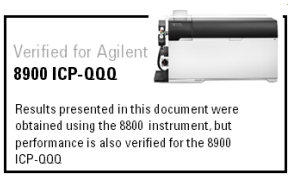
Since Cl has a high first ionization potential and is poorly ionized in the ICP plasma, ICP-MS sensitivity is usually low. This was overcome using a simple online sample preconcentration procedure. The drug-related compounds from a larger injection volume of human plasma were trapped on the preconcentration column, leading to a 25-fold improvement in the LOQ of Cl. This step broadens the application to metabolite profiling of low-dose pharmaceutical drugs containing Cl at sub mg/L levels.

References

1. Handbook of Hyphenated ICP-MS Applications, Agilent publication, 2012, 5989-9473EN
2. Balazs Klencsar, Lieve Balcaen, Filip Cuyckens, Frederic Lynen, Frank Vanhaecke, ***Analytica Chimica Acta*** 974, **2017**, 43–53
3. Naoki Sugiyama, Trace level analysis of sulfur, phosphorus, silicon and chlorine in NMP using the Agilent 8800 Triple Quadrupole ICP-MS, Agilent publication, 2013, 5991-2303EN

More Information

For a full account of this application, see Balazs Klencsar, Lieve Balcaen, Filip Cuyckens, Frederic Lynen, Frank Vanhaecke, Development and validation of a novel quantification approach for gradient elution reversed phase high-performance liquid chromatography coupled to tandem ICP-mass spectrometry (RP-HPLC-ICP-MS/MS) and its application to diclofenac and its related compounds, *Analytica Chimica Acta* 974, **2017**, 43–53, doi.org/10.1016/j.aca.2017.04.030.



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