White Paper

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Molecular Structure Solution of Impurities in Liquid Chromatography Assays using Microcrystal Electron Diffraction and High-Resolution Mass Spectrometry

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Abstract

Identification of impurities detected in chromatography assays according to the ICH-Q3 guidelines is a challenge because only trace quantities are available for analysis. Samples usually need to be collected from multiple HPLC runs making it time-consuming and expensive. Requiring orders of magnitudes less sample than traditional analytical techniques, microcrystal electron diffraction (microED) enables rapid crystal structure determination from nanoscale crystallites (~50–500 nm), but it can be challenging to assign all the atom types. High-resolution mass spectrometry (HRMS) provides an accurate *m*/*z* and offers complementary atom counts for candidate molecular formulae. The use of this very powerful combination of techniques is demonstrated on a model compound.

Keywords

Microcrystal electron diffraction (microED); high-resolution mass spectrometry (HRMS); molecular identification; HPLC assay; structure elucidation; pharmaceutical development; molecular formula assignment; trace-level impurities; Orbitrap; ICH-Q3.

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Introduction

The International Council for Harmonisation of Technical Requirements for Pharmaceuticals for Human Use (ICH) guideline Q3A states that, for new drug substances with typical daily doses of <1 g/day, organic impurities have to be identified if they amount to ≥ 0.1% in the chromatogram [1]. For new drug products (ICH-Q3B) these thresholds are typically 0.2%–0.5% [2]. Identification means determining the molecular structure of the impurity to assess its toxicity.

This requirement is a critical challenge in pharmaceutical development. It is particularly difficult because only small amounts of sample for a given impurity at a given relative retention time (RRT) in the chromatogram are available and obtaining sufficient amounts for spectroscopy can take dozens, if not hundreds, of repeat runs using preparatory high-pressure liquid chromatography (HPLC) to isolate the compound. Mass spectrometry (MS) and nuclear magnetic resonance spectroscopy (NMR) are most often used in tandem. However, because both techniques provide only fragmented information, it remains very challenging to solve the molecular puzzle unambiguously.

When new impurities at unknown RRT are identified later in development or in phase IV, the molecular structure is often key to identifying the root cause of the problem and time constraints are very high.

Microcrystal electron diffraction (microED) is an exceptionally powerful technique for determining the crystal structure of a material from microcrystals (i.e., crystalline powders). MicroED analyzes single, crystalline particles in the 50–500 nm range, much smaller than required for X-ray diffraction techniques. This eliminates the need to isolate larger quantities of material, dedicated crystal growth for single crystal X-ray diffraction (SCXRD), and requires many orders of magnitude smaller sample size than is needed for NMR, for instance.

Typical sample preparation for microED involves crushing crystalline powder into nanoscale particles. For impurity fractions obtained from HPLC, the solvent is evaporated to obtain the solids. For very small samples, the HPLC fraction can be deposited directly onto a transmission electron microscopy (TEM) grid for the solids to crystallize on, which can then be analyzed using microED. The challenge for this workflow is that the solids have to be amenable to crystallization during this process, as opposed to forming an amorphous material.

Although a very powerful technique, the atomic scattering factors are more similar for electron diffraction [3] than they are for X-ray diffraction [4]. This makes it more challenging to discern carbon atoms from nitrogen, oxygen, and fluorine (as well as between sulfur, phosphorus, and chlorine, and so on). Figure 1 shows a particle analyzed on a TEM grid by microED. Although the positions of the atoms are well-defined, which basically defines the overall shape of the molecule, crystal structure analysis can yield several feasible assignments of the elements.

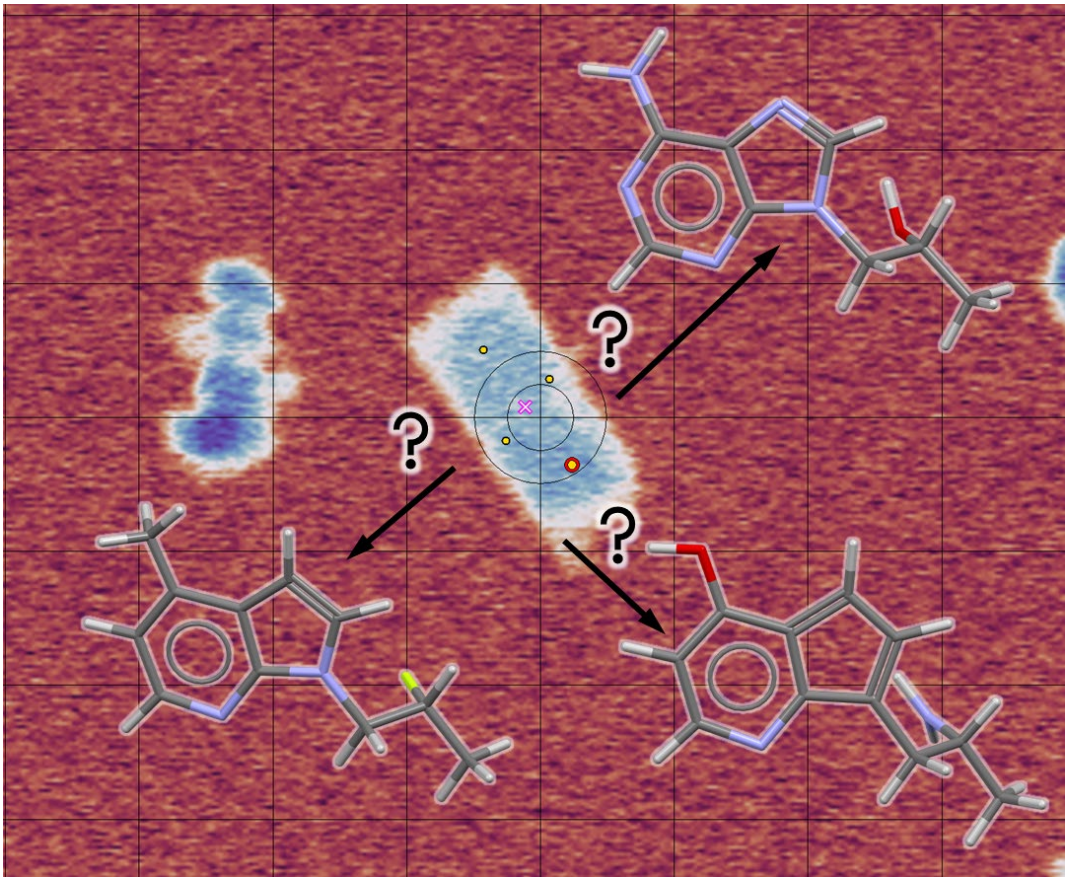

Figure 1. Electron microscope image showing a well-defined particle (blue rectangle), and potential molecular structures where atom assignments may be incorrect.

To help overcome the challenge of differentiating atoms with similar scattering factors in microED, we demonstrate high-resolution mass spectrometry (HRMS) as an orthogonal technique to provide an accurate mass-to-charge ratio (*m*/*z*), which, in turn, is used to determine a reasonable chemical formula that can be used when refining crystal structures from microED. While HRMS is a powerful technique for determining *m*/*z* and potential chemical formulae in itself, it is often very challenging to determine how all the fragments are interconnected and often yields several feasible candidates for the molecular structure. The combination of information from HRMS and microED will likely narrow the possibilities to a single structure, making the structure solution conclusive.

Recent successful client projects remain confidential. Here, we demonstrate the capability on a model compound.

Experimental

A model compound was purposely chosen such that the atom assignment would be ambiguous based solely on the microED structure. Ambiguity arises for compounds with a variety of oxygen- and nitrogen-containing moieties, particularly when they are heterocyclic.

(*R*)-1-(6-amino-9H-purin-9-yl)propan-2-ol is an intermediate in the production of tenofovir and was chosen as a good example.

A sample was taken directly from the reagent manufacturer's bottle without any attempts to improve crystallinity. It diffracted well and the structure was able to be solved using microED. The initial structure solution showed only one heteroatom, an oxygen, while the rest of the atoms were assigned as carbons (Figure *2*, left side).

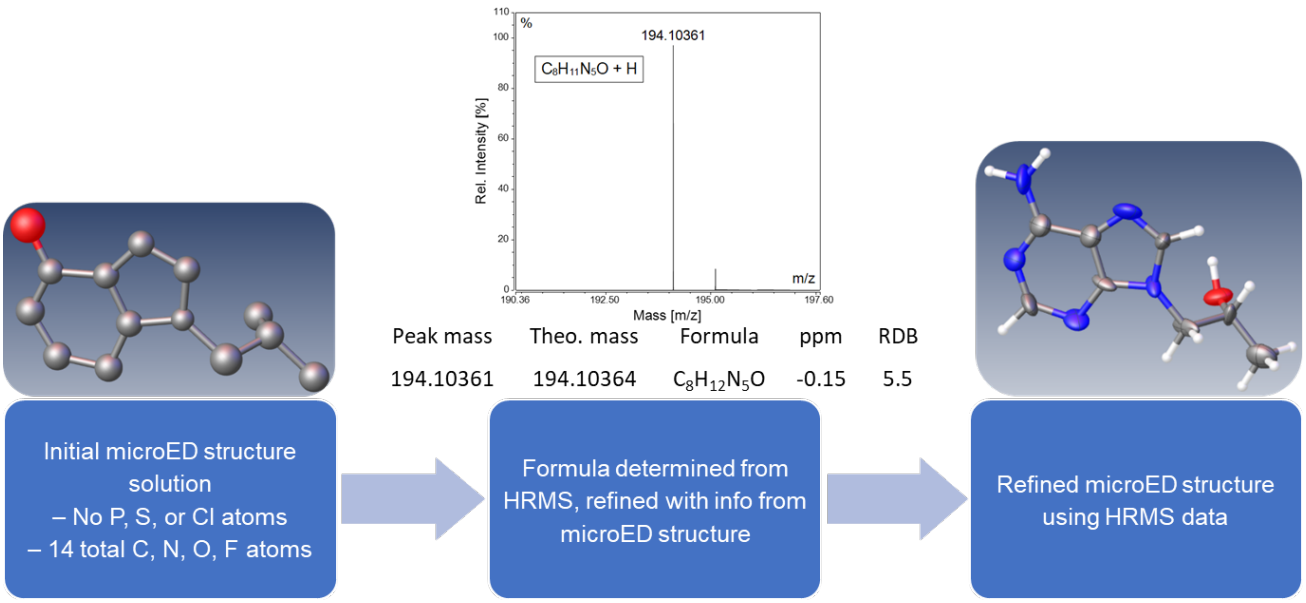

Figure 2. Workflow showing the initial microED structure solution (left), HRMS analysis to determine molecular formula (middle), and the fully refined crystal structure of the model compound (right).

A solution of the compound was analyzed by HRMS to obtain an accurate *m*/*z* value. A list of five possible chemical formulae with theoretical masses within the tolerance of the instrument (typically 0–4 ppm) was generated (Table 1). If HRMS was used as the only technique, comparison of experimental HRMS isotopic patterns with theoretical values and additional MS/MS fragmentation experiments would be required to reduce this list of possibilities. For example, $C_5H_{15}N_6{}^{35}Cl$ can be readily eliminated because the characteristic *m*/*z* patterns due to the ^{37}Cl isotope were not observed. The remaining formulae can be ranked by the closeness of their isotopic abundances to the theoretical pattern. However, it is immediately apparent that only one formula, $C_8H_{12}ON_5$ (observed in HRMS as the adduct $[C_8H_{11}ON_5 + H]^+$) is consistent with the microED observation because of the absence of P and S atoms, and the total of 14 C, N, O, and F atoms.

Note that the higher the mass of the compound, the longer the list of possible chemical formulae that can match the measured *m*/*z* within a given tolerance of mass accuracy. This makes the combination of HRMS and microED even more advantageous for compounds larger than the model compound. In pharmaceutical development, molecular masses in the range of 350–500 g/mol are more typical.

Table 1. List of molecular formulae within 4 ppm of the observed peak with *m*/*z* 194.10361, generated from HRMS data.

Potential Formula	Theoretical *m*/*z*	ppm difference (observed vs theoretical)	Number of suggested non-H atoms	Atoms consistent with microED?
$C_8H_{12}ON_5$	194.10364	−0.15	**14**	**Yes**
$C_9H_{17}F_2P$	194.10305	+2.90	12	No
$C_8H_{20}N^{32}S_2$	194.10317	+2.26	11	No
$C_4H_{15}ON_6P$	194.10395	−1.75	12	No
$C_5H_{15}N_6{}^{35}Cl$	194.10412	−2.66	12	No

An initial microED structure solution typically determines the total number of non-hydrogen atoms. This already greatly narrows the HRMS candidate list (Figure 2, middle). Sulphur and phosphorus could be eliminated because no atoms displayed the 3-dimensional bonding geometry of those atoms anywhere in the structure. The few remaining candidates can be further refined to produce a final, accurate crystal structure, where considerations such as bond lengths, hydrogen atom positions, anisotropic displacement parameters, and hydrogen bonding provide a final, accurate crystal structure (Figure 2, right side).

Conclusion

This case study on a model compound shows that the powerful combination of microED and HRMS can be used to conclusively solve crystal structures, and therefore molecular structures, using extremely small amounts of sample. Each technique on its own is powerful, but they complement each other's weaknesses.

Instrumentation

MicroED data was collected using an Eldico ED-1 electron diffractometer operating at an acceleration voltage of 160 kV (λ = 0.02851 Å) and a Dectris Quadro hybrid photon counting (HPC) detector.

HRMS data was collected using a Thermo Scientific Orbitrap Exploris 120 mass spectrometer operating in positive ion mode with an electrospray ionization (ESI) source.

Crystal structure solution

The crystal structure of (*R*)-1-(6-amino-9H-purin-9-yl)propan-2-ol, solved by MicroED, was deposited in the CSD (deposition number 2531479).

References

1. ICH harmonized tripartite guidelines (2006): Q3A(R2) Impurities in New Drug Substances; http://ich.org.
2. ICH harmonized tripartite guidelines (2006): Q3B(R2) Impurities in New Drug Products; http://ich.org.
3. L.-M. Peng, *Micron*, 1999, **30**, 625-648. doi:10.1016/S0968-4328(99)00033-5.
4. D. Waasmaier and A. Kirfel, *Acta Cryst*. 1995, **A51**, 416-431. doi:10.1107/S01087

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