Andrusenko, I., Hamilton, V., Mugnaioli, E., Lanza, A., Hall, C., Potticary, J., Hall, S. R., & Gemmi, M. (2019). The Crystal Structure of Orthocetamol Solved by 3D Electron Diffraction. *Angewandte Chemie - International Edition*, *58*(32), 10919-10922. https://doi.org/10.1002/anie.201904564

COMMUNICATION

The crystal structure of orthocetamol solved by 3D electron diffraction

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Abstract: Orthocetamol is a regioisomer of the well-known pain medication paracetamol and a promising analgesic and an anti-arthritic medicament itself. However, orthocetamol cannot be grown as single crystals suitable for X-ray diffraction, so its crystal structure has remained a mystery for more than a century. We report here the ab-initio structure determination of orthocetamol obtained by 3D electron diffraction, combining a low dose acquisition method and a dedicated single electron detector for recording the diffracted intensities. The structure is monoclinic, with a pseudo-tetragonal cell that favors multiple twinning on a scale of a few tens of nanometers. The successful application of 3D electron diffraction to orthocetamol introduces a new gold standard of total structure solution in all cases where X-ray diffraction and electron microscope imaging methods fail.

Difficult and long-lasting crystallographic problems are often connected with the intractability of growing large single crystals suitable for X-ray diffraction. Either the compound cannot crystallize and exists only in molecular form, or available crystals are ordered and coherent only in domains not larger than a few hundreds of nanometers. High resolution transmission electron microscopy (TEM) provides in these cases a way to bypass the absence of long-range order by directly imaging single macromolecules (CryoEM), or nanometric crystal domains with atomic resolution (HREM imaging). However, if the molecule is too small (<100 kDa) or the nanocrystals are particularly beam sensitive and do not stand the electron dose necessary for HREM, the crystallographic problem remains unsolvable by imaging methods.

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The first experimental protocol for acquiring 3D electron diffraction (3D ED) data was proposed by Kolb et al. in 2007[1]. This method allows sampling intensity data through the whole reciprocal space without knowing the crystallographic orientation of the sample, as is instead necessary for conventional in-zone ED patterns. 3D ED development was initially prompted by the need for a fast and efficient procedure for ED data collection on beam sensitive organic materials. Eventually, it transpired that this method also significantly reduced dynamical scattering, long considered the bottleneck of ED. 3D ED has therefore proven to be extremely efficient for the structure determination of many kinds of nanocrystalline compounds, including minerals[2], functional materials[3], zeolites[4] and MOFs[5]. More recently, the availability of a new generation of detectors able to count the arrival of single electrons have led to the development of extremely low dose acquisition protocols, based on continuous sample rotation[6] or on precession-assisted nanobeam electron diffraction coupled with scanning transmission electron microscopy (STEM) crystal tracking[7]. Such modes allowed the structure determination of extremely beam sensitive materials, like prions[8] and macromolecules[6b, 7, 9].

Continuous-rotation 3D ED, also referred to as MicroED, has been proven to be extremely efficient for structure determination of pharmaceuticals, without the need of growing large crystals.[10] However, to our knowledge all hitherto solved pharmaceuticals were test samples, whose structure had already been previously solved by X-ray diffraction. Orthocetamol (2-acetamidophenol) is an ortho-substituted regioisomer of paracetamol (4-acetamidophenol), one of the most widely used pharmaceuticals in the world (Figure S1). The ubiquity comes from the fact that paracetamol acts as an effective analgesic.[11] One major drawback of paracetamol however is the hepatotoxicity; overdoses of paracetamol remain the leading cause of liver damage across the UK/USA/Europe. This has led directly to an increase in research into related structures, particularly orthocetamol, which has been shown to demonstrate lower hepatotoxicity.[12]

Orthocetamol has also shown promise as an anti-arthritic treatment, a typical standard for inflammatory conditions, across a range of arthritic biomarkers, showing potential in not only halting progression of this disease, but in regulating the associated nociception.[13] Although both para- and meta-cetamol have solved crystal structures,[14] to-date there has been no structure of orthocetamol reported. Remarkably, orthocetamol was first reported in 1876,[15] i.e. before the discovery of X-rays. Therefore, this compound has existed for the entire history of crystallography with its structure unable to be determined either by single-crystal or powder methods.

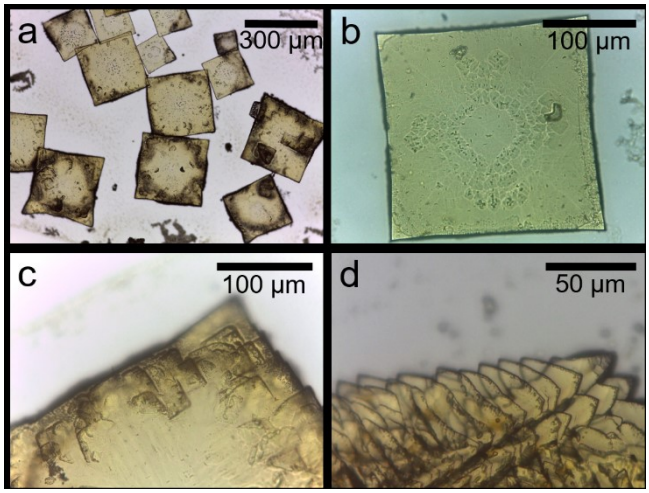

Figure 1. Orthocetamol platelets under light microscopy. a) Typical aspect of pale brown pseudo-squared orthocetamol aggregates. b) Example of a platelet with internal contrast features and evidences of sector boundaries along diagonals. It is evident that the square sides are not straight, as would be expected for a single crystal. c) Zoom of a platelet angle showing the actual occurrence of different overgrowing domains. d) Zoom of a broken platelet border that reveals the tile arrangement of thin domains.

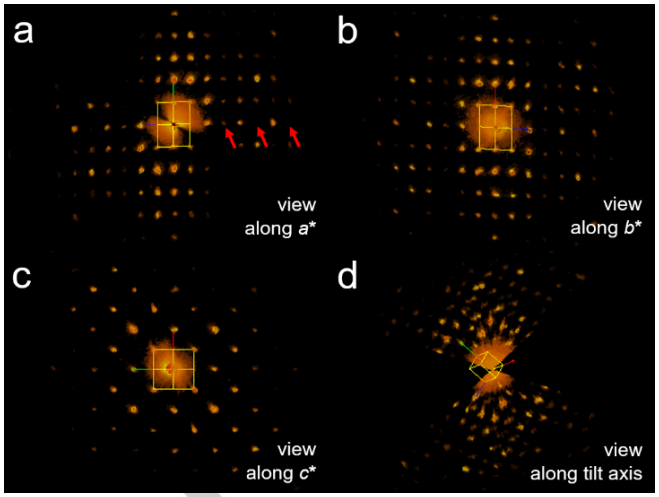

Figure 2. Orthocetamol diffraction volume reconstructed on the basis of 3D ED data. a) View along *a**.e Red arrows point columns of extinct reflections due to *c*-glide plane (*h0l* : *l* = 2n). Weak intensities are present due to residual dynamical effects and twinning. b) View along *b**. c) View along *c**, showing the typical chess-board pattern due to the lattice *C*-centering. d) View along the tilt axis of the acquisition, showing the sampled double-wedge of orthocetamol reciprocal space (from -60° to +60°). Cell edges are sketched in yellow. *a** vector is in red, *b** vector in green, *c** vector in blue. Note that these are projections of a 3D diffraction volume and not conventional 2D in-zone diffraction patterns.

Orthocetamol crystallizes as large quadrilateral platelets up to 300 µm in size (Figure 1). In fact, closer inspection reveals that the platelets are composed of a conglomeration of far smaller crystals (Figures S2 and S3). A few of these quadrilateral assemblies were therefore gently crushed and loaded on a carbon-coated Cu TEM grid. 3D ED data were collected at room temperature from eight fragments with size ranging from 500 nm to 2 µm (Figure S4). Data acquisition was performed stepwise using a parallel electron nanobeam of about 150 nm, while the crystal position was sequentially tracked in STEM mode.[7] Even fragments of few hundreds of nanometers show contrast features that suggest a certain degree of structural inhomogeneity.

The reconstructed 3D ED data delivered a pseudo-tetragonal primitive unit cell with parameters *a* = 7.4 Å and *c* = 13.7 Å. Such a cell would conveniently host four orthocetamol molecules, in apparent agreement with the multiplicity of point groups 4 and -4. Nevertheless, all attempts to solve ab-initio and refine orthocetamol structure with a tetragonal space group failed. Indeed, cells obtained from different 3D ED data showed significant deviations of cell angles, and symmetry determination was further complicated by the appearance of weak intensities for reflections that would eventually result extinct in the final correct symmetry. Such ambiguities are common for ED, but for orthocetamol they were aggravated by the systematic occurrence of stacking disorder and nanotwinning at the scale of a few cell repetitions.

On closer inspection, the data revealed that reflections *hhl* : *l* = 2n+1 were significantly weaker than reflections *hhl* : *l* = 2n. Such evidence led us to reduce the symmetry and re-index the 3D diffraction patterns by a *C*-centered orthorhombic cell with approximate parameters *a* = 10.5 Å, *b* = 10.5 Å, *c* = 13.7 Å (Figure 2). Potential extinctions pointed to space groups *Cccm* (66). This second trial allowed us to obtain an interpretable potential map where the orthocetamol molecule could be recognized. Yet, this pseudo-solution led to unphysical intermolecular distances and was evidently biased by too high symmetry constraints.

Symmetry was therefore reduced to the monoclinic space group *C*2/*c* (15). The structure of orthocetamol was then able to be solved ab-initio by direct methods and later refined by least-squares refinement (Figure 3a-c). All 11 non-hydrogen atoms were clearly recognizable in the potential map and were actually spotted by automatic routines. Atomic positions did not change significantly after least-squares refinement (see Figures S5 and S6). The choice of a monoclinic unit-cell was also supported by the successful indexing of the X-ray powder diffraction pattern, from which we obtained the refined lattice parameters: *a* = 10.5612(2) Å, *b* = 10.3856(2) Å, *c* = 13.7176(2) Å, *β* = 93.113(2) °, *V* = 1502.37(3) Å^3. Rietveld refinement on the same data and periodic DFT calculations additionally confirmed the structural model from ED (Figures S7 and S8).

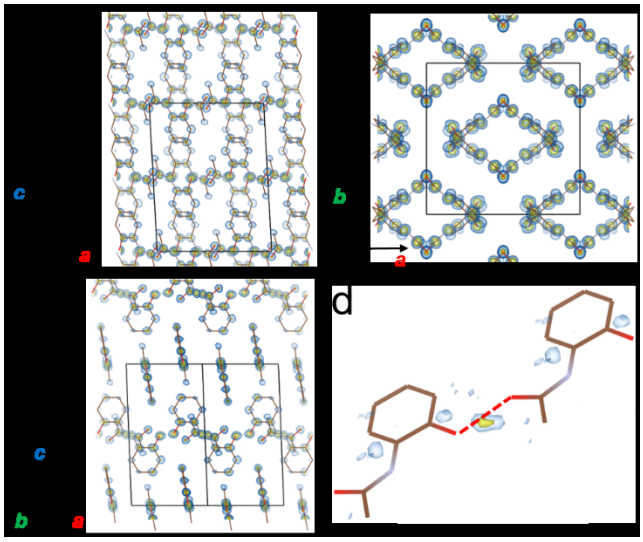

Figure 3. Orthocetamol structure and Fourier maps. a) View down [010] of orthocetamol structure superimposed to the Fourier map obtained after least-squares refinement against 3D ED data. Fourier map allows for a clear atomic resolution. b) View down [001] of orthocetamol structure. c) View down [110] of orthocetamol structure, showing the alternated layered packing of molecular ribbons. d) Two orthocetamol molecules belonging to the same ribbon, next to the main residual detected in the difference Fourier map (in yellow), after all hydrogen atoms were removed. The residual clearly indicates the position of the hydroxylic hydrogen atom involved in the hydrogen bond (dashed red line).

Our results show that the orthocetamol crystal structure consists of almost flat molecules that stretch along [110] and [1-10] in an alternated layered manner (Figure 3c). Notably, some hydrogen atoms were also visible in the difference Fourier map, even without dynamical structure refinement.[16] In particular, the hydroxylic hydrogen, involved in an hydrogen bond, showed a potential comparable with the non-hydrogen atoms of the structure and could in fact be determined ab-initio (Figure 3d).

Intermolecular hydrogen bonds involving the hydroxy and carbonyl groups of adjacent molecules form infinite ribbons. This packing motif is also seen for the chlorinated analogues 2-acetamido-4-chlorophenol and acetamido-5-chlorophenol, in the same space group but in less dense forms.[17]

The OH···O based supramolecular chain is a typical motif seen also in para- and metacetamol.[14] In all three paracetamol polymorphs, however, NH···OH interactions are also present and laterally connect adjacent chains to form 2D sheets.[14a-c] Spirally arranged NH···OH interactions are instead responsible for the formation of a 3D supramolecular network in the orthorhombic polymorph of metacetamol.[14d] The NH···OH interaction is absent in the monoclinic polymorph of metacetamol.[14e]

In the crystal structure of orthocetamol, chain alternation causes the NH donors to always point towards the aromatic rings and therefore they do not establish any relevant intermolecular interaction (Figure S6c). This molecular geometry seems then to prevent the hydroxyl group from being an effective acceptor of intermolecular H-bonds, and it is rather involved in an intramolecular NH···OH interaction.

Least-square refinement against ED data also confirmed the presence of twinning on a scale of about 150-200 nm, which produces a rotation of the structure of about 90° around the crystallographic axis *c*. Due to the resemblance of the cell dimensions *a* and *b*, pseudo-merohedric twinning mimics a tetragonal symmetry and brings intensities to otherwise extinct reflections.

To our knowledge, orthocetamol is the first purely organic pharmacological compound whose structure could be solved by ED and could not be solved by X-ray diffraction. The lack of structural solution via X-ray techniques is due to the fact that orthocetamol forms crystalline domains of only a few hundreds of nanometers, which are moreover affected by disorder and twinning. The occurrence of pseudo-symmetry further hinders structure analysis by conventional techniques. This structure determination of orthocetamol definitively shows that 3D ED can be used for solving ab-initio pharmaceutical structures that could not be unveiled by X-ray methods. We believe that orthocetamol will be the harbinger of widespread application of 3D ED for all pharmaceutical cases where structure determination by X-ray diffraction, TEM imaging and crystal structure prediction are not successful or simply cannot be applied. Additionally, as already displayed for MicroED protocol,[10c,d] 3D ED may prove to be a very fast and efficient method for the screening of synthetic products and intermediates, even when available only in scant quantity or as polyphasic or polymorphic mixtures.

Experimental Section

Orthocetamol (Sigma Aldrich, A7000) was recrystallized from cyclohexanol according to a previously published method.[18] Details of analytical methods and instrumentation are provided in the Supplementary Information.

Acknowledgements

S.R.H., J.P., C.H. and V.H. acknowledge the Engineering and Physical Sciences Research Council UK (grants EP/G036780/1 and EP/L015544/1), MagnaPharm, a collaborative research project funded by the European Union's Horizon 2020 Research and Innovation programme (grant No. 736899) and the Bristol Centre for Functional Nanomaterials and the Centre for Doctoral Training in Condensed Matter Physics. I. A., E. M., A. L. and M.G. acknowledge the Regione Toscana for funding the purchase of the Timepix through the FELIX project (Por CREO FESR 2014-2020 action).

Keywords: 3D electron diffraction • nanomaterials • pharmaceutical compound • structure determination • twinning

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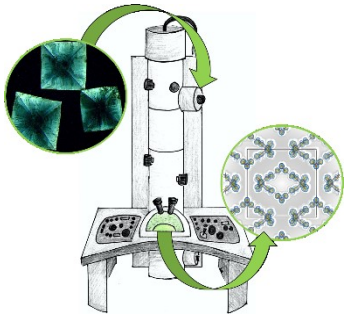
COMMUNICATION

For the first time 3D electron diffraction has been used to solve ab-initio the unknown crystal structure of a pharmaceutical compound that could not be solved with any other diffraction technique.



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