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# Challenges in the identification and quantification of an unknown impurity in chenodeoxycholic acid drug substance

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ABSTRACT

In 2018 the Amsterdam University Medical Centre decided to prepare chenodeoxycholic acid (CDCA) capsules (also known as pharmacy compounding) for patients with the genetic metabolic disease cerebrotendinous xanthomatosis (CTX) when the product with a marketing authorization was commercially unavailable for patients. However, after reanalysis, unknown impurities were identified in the CDCA active pharmaceutical ingredient (API) using thin-layer chromatography from the European Pharmacopoeia (Ph.Eur.) monograph. Therefore, the API did not comply with the Ph.Eur. specifications for related substances and as a result, pharmacy compounding was halted and an investigation was initiated to identify and quantify the unknown impurities. Meanwhile, a second CDCA API was sourced from another manufacturer. However, this API also appeared to contain an unknown impurity. This impurity could be identified as a dimer of CDCA using reversed phase liquid chromatography mass spectrometry. Since the Ph.Eur. at the time did not describe a suitable analytical method for the quantification of this new impurity, a high pressure liquid chromatography with differential refractometer (HPLC-RI) method was developed to quantify the dimer. Subsequently, in 2019, a new draft version of the CDCA Ph.Eur. monograph was published, including the dimer as a new impurity together with a HPLC-RI method for its identification and quantification. The CDCA-dimer is classified as non-toxic and permitted in the CDCA API up to a maximum of 0.5 %. Because the API complied with the updated Ph.Eur. specifications, pharmacy compounding of CDCA capsules could be resumed.

## 1. Introduction

Chenodeoxycholic acid (CDCA) is a primary bile acid and the cornerstone of treatment of patients with cerebrotendinous xanthomatosis (CTX) (Federico and Gallus 2003). CTX is a rare genetic metabolic disease caused by mutations in the *CYP27A1* gene. Patients with CTX have reduced or absent endogenous levels of the primary bile acids CDCA and cholic acid and, consequently, formation and accumulation of toxic bile acid intermediates and cholestanol in tissues (Federico and Gallus 2003). The disease has a variable course, mostly characterized by infantile-onset diarrhea, followed by cataract and tendon xanthomas in childhood, with later onset of neurologic dysfunction including

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psychiatric disturbances, cerebellar symptoms, neuropathy and dementia (Federico and Gallus 2003). When treatment with CDCA is started before neurological symptoms appear, neurological complications and other symptoms can be prevented (Stelten et al., 2019). The availability of CDCA as a medicinal product is therefore important to CTX patients.

In the Netherlands, CDCA Leadiant capsules authorized by the EMA are unavailable for CTX patients since CDCA Leadiant is not included in the Dutch basic healthcare package and is therefore not reimbursable under the Healthcare Insurance Act (Sheldon 2018). To assure continuation of patient treatment, the department of pharmacy and clinical pharmacology of the Amsterdam University Medical Centre (Amsterdam UMC) developed CDCA capsules from CDCA API through pharmacy compounding, also known as pharmacy preparation (Polak et al., 2021; Sheldon 2018). Pharmacy compounding is the preparation of a medicinal product for an individual patient by a pharmacy. Compounding started in 2018 with CDCA API from an Asian manufacturer. As CDCA API could only be procured from outside the European Union (EU), QC testing within the EU was necessary to assure compliance with EU requirements.

A reliable and qualified supply chain is important to assure adequate manufacturing, storage and distribution. The requirements for the manufacturing and distribution of APIs are described in EU Good Manufacturing Practices (GMP) and Good Distribution Practices (GDP) guidelines *Basic Requirements for Active Substances used as Starting Materials (European Commission 2014)* and *Guidelines on principles of Good Distribution Practice of active substances for medicinal products for human use* (European Commission 2015), respectively. It is essential that API manufacturers and suppliers comply with these EU GMP and GDP requirements and possess the required licenses (European Parliament and Council of the European Union 2017; European Parliament and Council of the European Union 2022).

Furthermore, APIs have to comply with general and (if available) individual monographs of the European Pharmacopoeia (Ph.Eur.). For CDCA, individual Ph.Eur. monograph 1189 is available (EDQM 2017; EDQM 2022). General monograph 2034 *Substances for pharmaceutical use* is the starting point for APIs and describes which other general chapters and monographs are applicable (EDQM 2021). For APIs from animal origin used for oral pharmaceutical preparations such as CDCA, other applicable general Ph.Eur. monographs and chapters include: chapter 5.1.7 *Viral safety*, chapter 5.10 *Control of impurities in substances for pharmaceutical use*, monograph 1483 *Products with risk of transmitting agents of animal spongiform encephalopathies*, chapter 5.4 *Residual solvents*, chapter 5.20 *Elemental impurities*, chapter 5.9 *Polymorphism*, and chapter 5.1.4 *Microbiological quality of non-sterile pharmaceutical preparations and substances for pharmaceutical use* (EDQM 2021).

One of the main challenges was the Quality Control (QC) testing of the CDCA API. During QC testing on related substances (the Ph.Eur. test description for impurities) with thin-layer chromatography (TLC) according to Ph.Eur., no impurities were initially found. However, after reanalysis using the same TLC method, the CDCA API did in fact contain unknown impurities above the specified limits in the monograph. Based on this result, Amsterdam UMC initiated a recall at patient level and the pharmacy compounding was halted. Treatment with the Amsterdam UMC product was ceased and an investigation on the unknown impurities was started (Sheldon 2018). Meanwhile, the Amsterdam UMC pharmacy investigated another CDCA API source from Asia, but also this API contained an unknown impurity above the specified limit, as tested with TLC, whereafter the investigation continued. This article describes the challenges in QC of CDCA API and the identification and quantification of an unknown impurity in order to assess if the CDCA API can safely be used in pharmacy compounding to continue patient treatment.

**Table 1**
Tests and specifications of CDCA API according to Ph.Eur. individual monograph 1189 (2017) and general monograph 2034 Substances for pharmaceutical use.

| Test description | Specification | Method | Reference |
|---|---|---|---|
| Appearance | White or almost white powder | Visual | Ph.Eur. 1189 |
| Specific optical rotation | +11.0° to +13.0° | Ph.Eur. 2.2.7 | Ph.Eur. 1189 |
| Identification (IR) | Positive | Ph.Eur. 2.2.24 | Ph.Eur. 1189 |
| Related substances (TLC) | -Ursodeoxycholic acid: Max. 1 % (impurity A)<br>-Cholic acid: Max. 0.5 % (impurity B)<br>-Lithocholic acid: Max. 0.1 % (impurity C)<br>-Any other spot: Max. 0.25 %<br>-Two clearly separated principal spots | Ph.Eur. 2.2.27 | Ph.Eur. 1189 |
| Loss on drying | Max. 1.5 % | Ph.Eur. 2.2.32 | Ph.Eur. 1189 |
| Sulphated ash | Max. 0.1 % | Ph.Eur. 2.4.14 | Ph.Eur. 1189 |
| Assay (dried) | 99.0 % - 101.0 % | Ph.Eur. 2.2.20 | Ph.Eur. 1189 |
| Polymorphism* | 166 °C ± 3 °C | Ph.Eur. 2.2.34 | Ph.Eur. 5.9 |
| Residual solvents* | Complies to Ph.Eur. 5.4 | Ph.Eur. 2.4.24 | Ph.Eur. 5.4 |
| Microbiology* | -TAMC: NMT $10^3$ CFU/g<br>-TYMC: NMT $10^2$ CFU/g | Ph.Eur. 2.6.12 | Ph.Eur. 5.1.4 |

* only for second API.

## 2. Materials and methods

### 2.1. CDCA API material and specifications

CDCA API was procured from two different Asian manufacturers (confidential). Both APIs were tested according to Ph.Eur. individual monograph CDCA 1189 (2017) (EDQM 2017). For analysis of the second API, additional tests were included according to general monograph 2034 (EDQM 2021). The specifications can be found in table 1. QC testing was outsourced to an external GMP certified EU contract laboratory (confidential).

### 2.2. Ph.Eur. analytical methods for related substances of CDCA API

Related substances, as described in the Ph.Eur., are organic impurities with a similar structure compared to the API. According to Ph.Eur. general text 5.10 *Control of impurities in substances for pharmaceutical use,* the impurities section of the monograph describes all impurities that to some extent may be present in the API and are known to be detected by the tests described in the concerning individual monograph (EDQM 2019b). Related substances of CDCA are analyzed according to the methods described in Ph.Eur. CDCA individual monograph 1189 (EDQM 2017; EDQM 2022). At the start of the investigation the 2017 version of the monograph required testing of related substances by means of TLC (EDQM 2017). In July 2019 a new draft monograph was published in Pharmeuropa 31.3 (EDQM 2019a) by the European Directorate for the Quality of Medicines and HealthCare (EDQM), which came into effect in January 2022 with Ph.Eur. edition 10.6 (EDQM 2022). The TLC test for related substances was replaced by two high pressure liquid chromatography methods with differential refractometer (HPLC-RI) (EDQM 2022).

According to Ph.Eur. general monograph 2034 *Substances for pharmaceutical use* other potential impurities that are not described in the individual (CDCA) monograph need to be reported, identified and qualified with reporting, identification and quantification thresholds based on the maximum daily dose (EDQM 2019b; EDQM 2021). For

**Table 2**
Related substances specifications CDCA Ph.Eur. monograph 1189, 2017 vs. 2022. (EDQM 2017; EDQM 2022).

| Ph.Eur. Impurity | Description | Ph.Eur. 2017 monograph (TLC) | Ph.Eur. 2022 monograph (HPLC-RI) |
|---|---|---|---|
| Impurity A | Ursodeoxycholic acid | Max. 1 % | * |
| Impurity B | Cholic acid | Max. 0.5 % | Max 0.25 % |
| Impurity C | Lithocholic acid | Max. 0.1 % | * |
| Impurity D | Ursocholic acid | * | * |
| Impurity E | Deoxycholic acid | * | * |
| Impurity F | 3α-hydroxy-7-oxo-5β-cholan-24-oic acid | * | * |
| Impurity G | Methyl 3α,7β-dihydroxy-5β-cholan-24-oate | * | * |
| Impurity H | 3α,7β-dihydroxy-12-oxo-5β-cholan-24-oic acid | Not described | Max. 0.2 % |
| Impurity I | 3α-[(3α,7α-dihydroxy-24-oxo-5β-cholan-24-yl)oxy]−7α-hydroxy-5β-cholan-24-oic acid (CDCA dimer) | Not described | Max. 0.5 % |
| Other / Unspecified impurities | NA | Max. 0.25 % | Max. 0.10 % |
| Total impurities | NA | Not described | Max. 1.5 % |

* These impurities are described in the monograph but not included as tests. If present at a sufficient level, they would be detected by one or other of the tests in the monograph.

CDCA the maximum daily dose is 1 g, and therefore the reporting threshold is >0.05 %, the identification threshold is >0.10 % and the qualification threshold is >0.15 % (EDQM 2021).

#### 2.2.1. Related substances analysis in 2017 CDCA monograph with TLC method

The TLC test for related substances is described in Ph.Eur. CDCA individual monograph 1189 (2017) (EDQM 2017). The following solutions are applied to a TLC plate: test solution a: 40 mg/ml CDCA API, test solution b: 4 mg/ml CDCA API, reference solution a: 4 mg/ml CDCA standard, reference solution b: 0.04 mg/ml lithocholic acid standard (=0.1 % of test solution a), reference solution c: 0.4 mg/ml ursodeoxycholic acid standard (=1 % of test solution a), reference solution d: 0.2 mg/ml cholic acid standard (=0.5 % of test solution a), reference solution e: 0.01 mg/ml CDCA API (=0.25 % of test solution a), and reference solution f: 0.4 mg/ml CDCA standard + 0.4 mg/ml ursodeoxycholic acid standard. Any spots in test solution a corresponding to lithocholic acid (Impurity C), ursodeoxycholic acid (Impurity A) and cholic acid (Impurity B), cannot be more intense than the spot in the corresponding reference solutions b, c and d. Meaning a maximum of 0.1 %, 1 % and 0.5 % of the respective impurities. Any other spots in test solution a cannot be more intense than the spot in reference solution e. Meaning a maximum of 0.25 % for other impurities described in the impurities section of the monograph (Impurities D, E, F and G). Reference solution f should show clearly separated spots. The impurities are also described in Table 2 (EDQM 2017).

#### 2.2.2. Related substances analysis in 2022 CDCA monograph with HPLC-RI methods

In the HPLC-RI test, related substances (impurities B, H and I) are specified with limits of 0.25 %, 0.2 % and 0.5 %, respectively (EDQM 2022). Unspecified impurities are allowed at a maximum of 0.10 % and total impurities at a maximum of 1.5 % (EDQM 2022). Impurities H and I, and the maximum for total impurities are new in the 2022 monograph (EDQM 2022). The specifications for related substances described in the 2017 and the revised 2022 monograph are compared in Table 2 (EDQM 2017; EDQM 2022). Structural formulas of all described related substances can be found in Supplementary Material 1 (EDQM 2022).

**Table 3**
Initial QC results of first API.

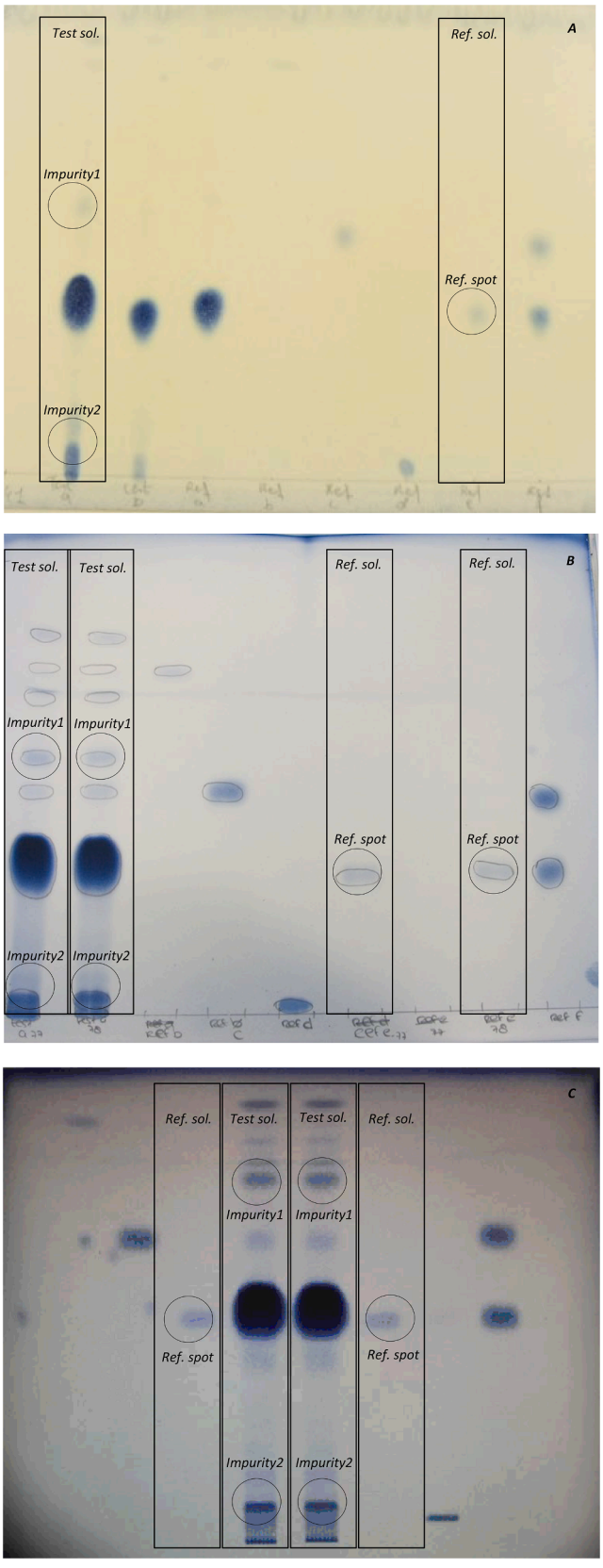
| Test description | Specification | Results |
|---|---|---|
| Appearance | White or almost white powder | White or almost white powder |
| Specific optical rotation | +11.0° to +13.0° | +11.27° |
| Identification (IR) | Positive | Positive |
| Related substances (TLC) | -Ursodeoxycholic acid: Max. 1 % (impurity A)<br>-Cholic acid: Max. 0.5 % (impurity B)<br>-Lithocholic acid: Max. 0.1 % (impurity C)<br>-Any other spot: Max. 0.25 %<br>-Two clearly separated principal spots | Complies |
| Loss on drying | Max. 1.5 % | 1.1 % |
| Sulphated ash | Max. 0.1 % | 0.03 % |
| Assay (dried) | 99.0 % - 101.0 % | 100.0 % |

### 2.3. Analytical methods for impurity identification and quantification

Impurity identification was performed in collaboration with the Leiden University Medical Center (LUMC) laboratory. Given the fact that the unknown impurity was observed during TLC based QC testing, TLC was employed as a first separation dimension. In the first instance, the MS was operated in scan mode to identify differences between blank and sample analysis. The spot of the unknown impurity was scraped from the analyzed TLC plates and dissolved in 1.0 mL acetone. The sample was briefly vortexed and sonicated. After centrifugation (5 min at 16,100 x *g*), the supernatant was transferred to a glass vial and dried under a gentle stream of nitrogen. The sample was reconstituted in 40 % MeOH: water for subsequent analysis. For the hydrolysis experiment, 20 µL of the unknown impurity in 40 % MeOH:water was combined with 1 µL 10 M sodium hydroxide solution, the sample was vortexed and kept at 60 °C for 30 min. After the sample had cooled down it was neutralized using 10 % formic acid in water and subsequently used for analysis.

The samples were analyzed by reversed phase liquid chromatography mass spectrometry (RP-LC-MS/MS). A Shimadzu LC system consisting of two LC-30AD pumps, a SIL-30AC autosampler, and a CTO-20AC column oven (Shimadzu, 's-Hertogenbosch, The Netherlands) was coupled to a Sciex TripleTOF 6600 equipped with an electrospray ionisation (ESI) source. 5 µL sample was injected. Separation was achieved on a Phenomenex Kinetex C18 column (1.7 µm, 100 Å, 50 × 2.1 mm; Phenomenex, Utrecht, The Netherlands) protected with a Phenomenex C8 precolumn, that were held at 50 °C. Analytes were eluted using 0.1 % formic acid in water (A) and 0.1 % formic acid in MeOH (B) at a flow rate of 0.4 mL/min using the following gradient: 5 % B at 0 min, constant at 5 % B until 1 min, linearly increased to 100 % B at 11 min, held constant at 100 % B until 16 min. The ion source temperature was set to 500 °C, the ion spray voltage was −4500 V, ion source gas 1, ion source gas 2 and curtain gas were all set to 30. The declustering potential was set to −80 V, masses between $m/z$ 100 and 1000 were monitored. For MS/MS analysis, the ion $m/z$ 765.6 was isolated and fragmented using a collision energy of −85 V and a collision energy spread of 25 V.

Identification was followed by quantification. At that time the revised Ph.Eur. monograph including the HPLC-RI methods was not yet published. Because the TLC method is not specific, the second API manufacturer developed an HPLC-RI test method for related substances to identify and quantify potential impurities (analytical method details are confidential).




*(caption on next column)*

**Fig. 1.** TLC results of first CDCA API, tested according to Ph.Eur. monograph 1189 (2017). Full TLC plates are shown, only boxed chromatograms are relevant for the results described in the paper. A: laboratory 1 (single batch): The left square is test solution a with impurities 1 and 2 circled. The right square is reference solution e, with the reference spot for 0.25 % circled. B: laboratory 2 (two batches): The left 2 squares are test solution a for 2 batches, with impurities 1 and 2 of >0.25 % circled. The right 2 squares are the corresponding reference solution e, with the reference spots for 0.25 % circled. C: laboratory 3 (two batches): The 2 middle squares are test solution a for 2 batches, with impurities 1 and 2 of >0.25 % circled. The outer 2 squares are the corresponding reference solution e, with the reference spots for 0.25 % circled.

## 3. Results

### 3.1. QC results Ph.Eur. of first API

The test results of the first API according to Ph.Eur. CDCA individual monograph 1189 (2017), as described in chapter 2.1, all complied and are shown in Table 3.

### 3.2. Related substances results

#### 3.2.1. TLC

Initially, the laboratory reported that the first API complied to the TLC test for related substances. However, TLC analyses performed by two other laboratories showed that two impurities were found above the specification of max. 0.25 %, called impurity 1 and impurity 2 in Fig. 1. Due to the impurity issue, the second API was initially only tested for related substances. TLC results also showed an unknown impurity, called impurity 3, see Fig. 2. The impurity in the second API shows approximately the same Rf value (retention factor, ratio of the distance of the compound to that of the mobile phase) as compared to impurity 1 in Fig. 1. Although the Ph.Eur. monograph is for APIs and not suitable for drug products, an additional TLC test was performed comparing the two APIs with the commercial CDCA drug product. An unknown impurity also appeared to be present in the authorized product, called impurity 4, see Fig. 3. Impurity 1, 3 and 4 seem to be the same impurity and knowing this impurity exists in all tested compounds, investigation continued on the second API which showed a better impurity profile with only this one unknown impurity. The other impurity (impurity 2 in Fig. 1) in the first API was not further investigated, as the main goal was to continue patient treatment as soon as possible.

#### 3.2.2. Identification of impurity by RP-LC-MS/MS

As shown in Fig. 4A-C a differentiating signal was observed at 11.22 min with a $m/z$ of 765.57. Subsequently, tandem mass spectrometry analysis (MS/MS) of $m/z$ 765.57 was carried out and two fragments were observed, one at $m/z$ 391.29 and one at $m/z$ 373.28 (Fig. 4D). Based on the obtained MS data it was speculated the unknown impurity concerns an ester dimer of CDCA (Fig. 5). Based on this assumption, a charge remote fragmentation was proposed leading to a neutral loss and a keto-component of $m/z$ 391.25, subsequent water loss will then lead to fragment $m/z$ 373.24 (Fig. 5). To further test this hypothesis alkaline hydrolysis was carried out. As shown in Fig. 6, hydrolysis led to a complete disappearance of the signal related to the impurity ($m/z$ 765.57 peak at 11.22 min) while a CDCA peak ($m/z$ 391.29 peak at 9.33 min.) was formed. Subsequently, it could be concluded that the observed impurity is an ester dimer of CDCA.

#### 3.2.3. HPLC-RI results

Using the in-house HPLC-RI method from the second API manufacturer, the percentage of the dimer could be accurately determined. Fig. 7 shows an example HPLC-RI chromatogram including the dimer impurity. The second API batch containing the impurity as tested with the TLC method, showed a dimer percentage of 0.77 %, confirming the out of specification TLC result. Two other available CDCA API batches at the

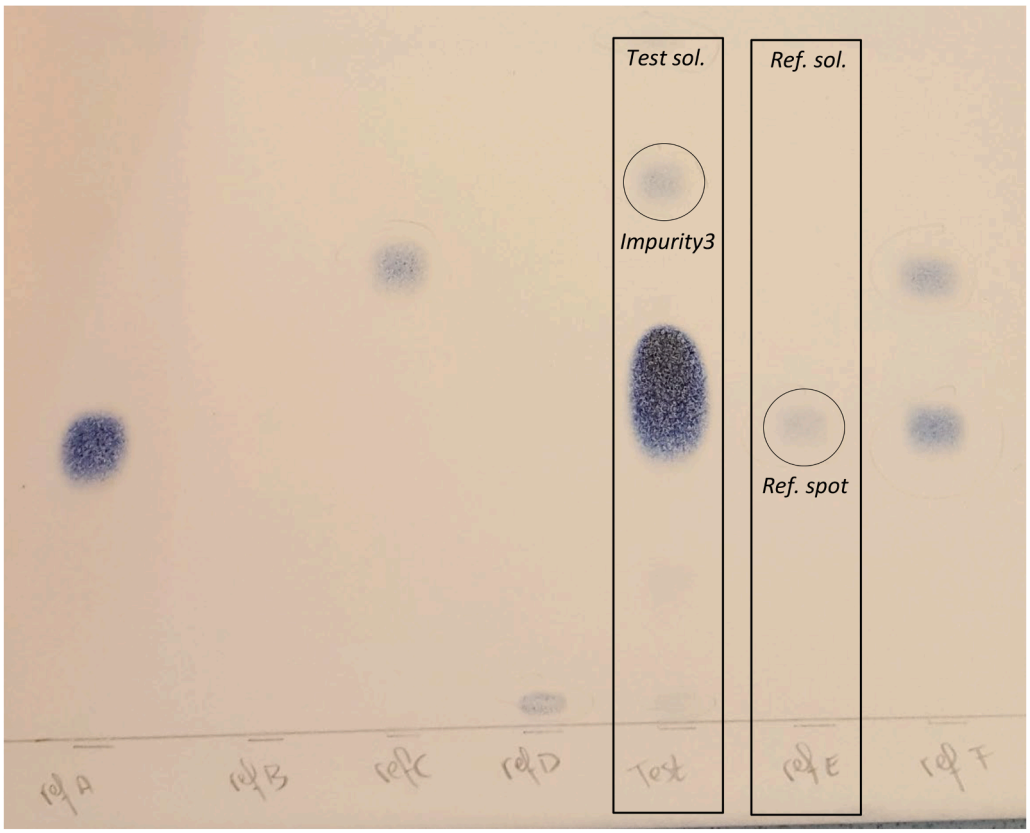



**Fig. 2.** TLC plate of second CDCA API, tested by contract laboratory according to Ph.Eur. monograph 1189 (2017). The left square is test solution a with impurity 3 circled. The right square is reference solution e, with the reference spot for 0.25 % circled. Full TLC plate is shown, only boxed chromatograms are relevant for the results described in the paper.

manufacturer, were also analyzed by the manufacturer and showed dimer percentages below 0.5 %.

### 3.3. QC results Ph.Eur. of second API

After successful quantification using the HPLC-RI method from the API manufacturer, the new draft version of Ph.Eur. CDCA individual monograph 1189 was published including the new HPLC-RI methods and the dimer impurity, called Impurity I, with a limit of maximum 0.5 %. The batch with the lowest percentage of the dimer impurity, as analyzed by the manufacturer, was retested by a contract laboratory using the draft Ph.Eur. HPLC-RI method, and the result was confirmed: the dimer impurity level complied to the draft monograph (Impurity $I \leq$ 0.5 %). All other tests from the monograph also complied, see Table 4 for full release results.

## 4. Discussion

In this article the challenges in quality control of CDCA API were described. Our investigation showed that we were able to identify and quantify a hitherto unknown impurity. Furthermore, our results showed that it was possible to obtain a CDCA API which complied with the specifications of the Ph.Eur. suitable for providing CTX patients with pharmacy compounded CDCA capsules.

The quantification of related substances with the TLC method described in the 2017 Ph.Eur. monograph proved unreliable. The interpretation of the generated spots is subjective which can lead to different results. In our case, results of the first analysis of the first API were interpreted as compliant, but results after reanalysis as non-compliant. The concentrations of the related substances in the API were near the allowed limits which makes it difficult to compare the obtained spots.

Another challenge was that the updated Ph.Eur. monograph with HPLC-RI analysis of the related substances was not yet in effect at the time these analyses were performed and that compliance with the 2017 monograph version was still obligatory. As previously explained in chapter 2.2, Impurity I was not yet described in the 2017 monograph version, and as a consequence, the limit of the dimer should not exceed the specification of maximum 0.25 % (EDQM 2017).

According to Ph.Eur. general monograph 2034 *Substances for pharmaceutical use*, if "the individual monograph does not provide suitable control for a new impurity, a suitable test for control must be developed and included in the specification for the substance" (EDQM 2021). A draft of the updated monograph was published in 2019 (EDQM 2019a), including a more suitable HPLC-RI method for the identification and quantification of related substances, as it is much more sensitive and specific than TLC. HPLC-RI analysis of Impurity I was therefore added to our QC specifications for related substances, with a limit of maximum 0.5 %, equal to the updated draft monograph. We considered it justified to use the API with ≤0.5 % Impurity I in our pharmacy compounding, also because the dimer was found in the authorized product which was already used for long term by patients.

Pharmacy compounding of CDCA capsules was resumed with the second API that complied with our specifications. Consequently, the supply of compounded CDCA capsules to our patients was resumed as from January 2020 (Sheldon 2020). In January 2022, 2,5 years after first publication of the draft, the updated CDCA monograph became effective. From that moment onward, all API batches were tested in accordance with this updated monograph.

However, we identified a gap in procedural guidance regarding the handling of situations where a new draft monograph is published, but not yet effective. Revisions to Ph.Eur. monographs are typically

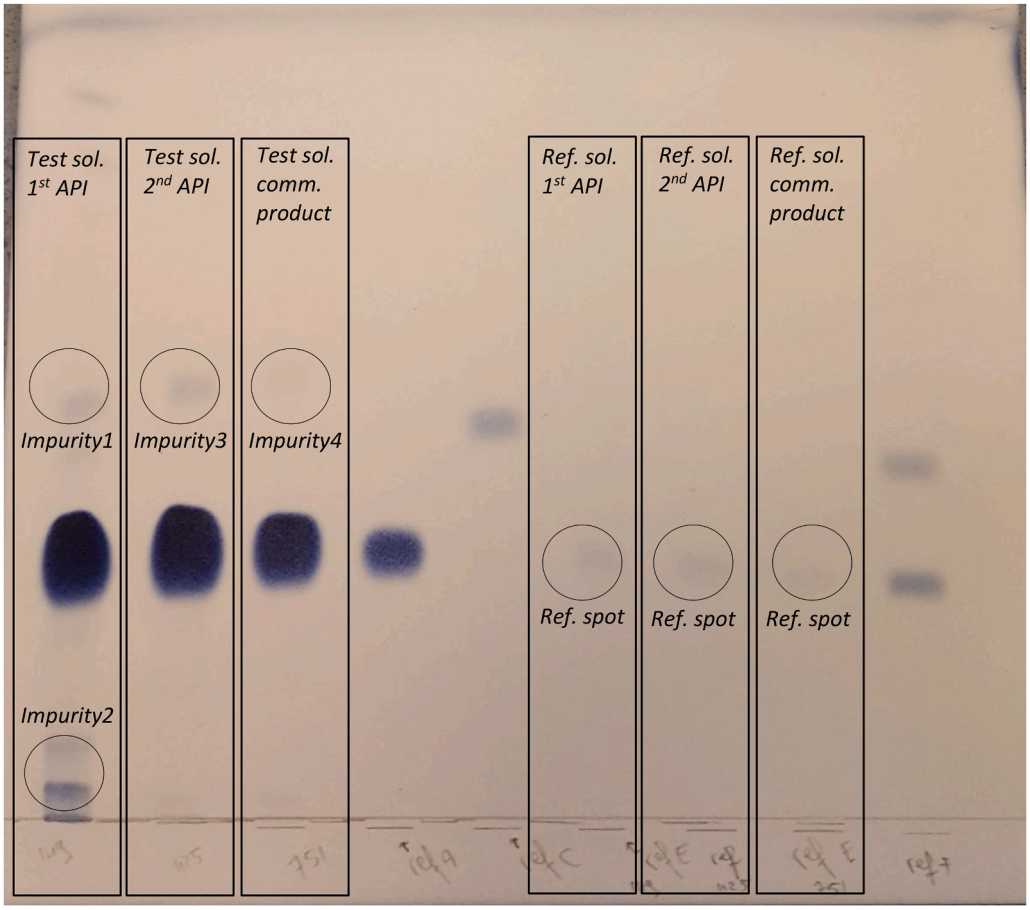



**Fig. 3.** TLC plate of both APIs and authorized CDCA capsule, tested by contract laboratory according to Ph.Eur. monograph 1189 (2017). The left squares are test solution a of first API, second API, and commercial product respectively, with the impurities circled. The right squares are their respective reference solution e, with the reference spot for 0.25 % circled. The results for the commercial drug was within specification. However, no correction was applied for using a final product instead of pure API. The actual amount of CDCA API tested is probably less due to the additives in the capsule. Full TLC plate is shown, only boxed chromatograms are relevant for the results described in the paper.

intended to improve the quality control of APIs and are often initiated by API manufacturers or end-users. In the case of CDCA API, it is plausible that other stakeholders encountered similar issues with the dimer impurity, leading to the development of a new draft version of the CDCA monograph that included improved methods for controlling related substances. The implementation of new monographs is inherently time-consuming due to the necessary consultations and the need for reference materials required for the proposed methods. Perhaps parts of our investigation could have been prevented if this information had been openly accessible earlier than at publication of the draft. This example shows that it is advisable to periodically check for new or updated draft monographs. The EDQM helpdesk is available for more information and their work program is available for Ph.Eur. stakeholders.

Another important challenge we faced was that the CDCA API was not available through our regular and approved API suppliers, necessitating procurement from sources outside the EU. Generally, procuring APIs from within the EU is much simpler due to the compliance with EU GMP guidelines, availability of EU GMP certificates and conducted QC within the EU. However, nowadays many APIs originate from outside the EU, meaning reanalysis within the EU is needed where issues can arise. In our case, the second API manufacturer was essential in our investigation by developing analytical methods for the identification and quantification of the dimer impurity. This underlies the importance of a reliable supply chain and good contact with API suppliers, which is easier to maintain within the EU.

Also, both API manufacturers did not possess a Certificate of Suitability to the monographs of the European Pharmacopoeia (CEP). A CEP certifies that an API is adequately controlled by the tests described in the relevant monograph, which are based on a specific synthesis and starting materials. Variations in the manufacturing process can lead to other impurities which need to be determined and controlled. Not all manufacturers offer APIs with a CEP, it is not obligatory and comes with costs and a rigorous regulatory process. If a CEP is not available, it is necessary to assess the suitability of the individual monograph tests by evaluating the synthesis route. The Drug Master File (DMF) or Active Substance Master File (ASMF) from the API manufacturer, which contains detailed synthesis information, can be helpful in this assessment, although not every manufacturer will be willing to share this detailed information about their production.

For CDCA API, the pharmacopoeial information on the synthesis route was published in the German commentary to the Ph.Eur. 4.00 (Arzneibuch-Kommentar 2004). This text describes how CDCA can be derived from goose- or bovine bile or can be synthesized from cholic acid. The dimer impurity likely arises as a condensation product during CDCA synthesis. Also, bile, being a mixture of various bile acids, varies in composition depending on its origin (Hofmann and Hagey 2014). In the purification and production process of bile acids for the synthesis of bile acid APIs, it is inevitable that other bile acids will be present to some extent, either from the source material or as a byproduct from synthesis. The Ph.Eur. monograph takes this into account by setting maximum limits for various bile acids impurities. Because the unknown impurity was detected by the test for related substances, we hypothesized from

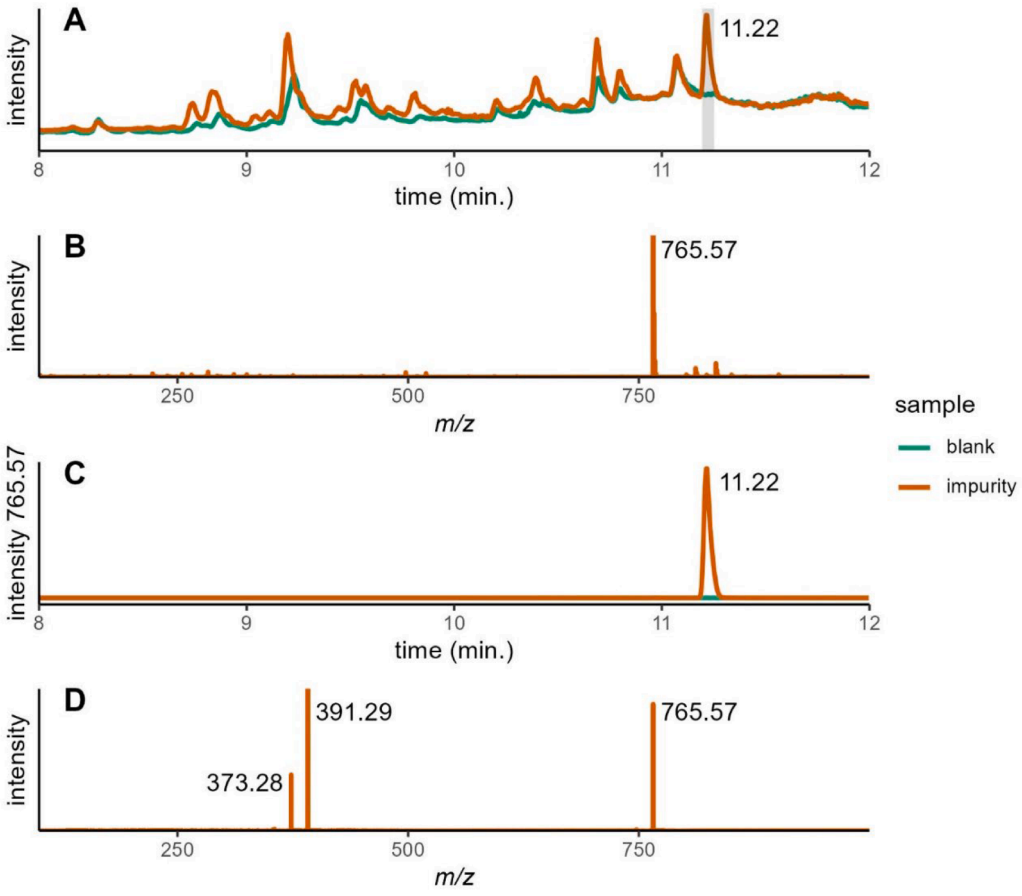



**Fig. 4.** RP-LC-MS/MS analysis of the impurity-containing TLC band. A: Total ion chromatogram of RP-LC-MS/MS analysis comparing blank versus impurity-containing band, with the difference the peak at $T = 11.22$ min. B: Negative mode electrospray ionization mass spectrum of peak at 11.22 min. C: Extracted ion chromatogram of *m/z* 765.57 (dimer) comparing blank and TLC impurity band. D: Tandem mass spectrometry analysis of *m/z* 765.57 (dimer).

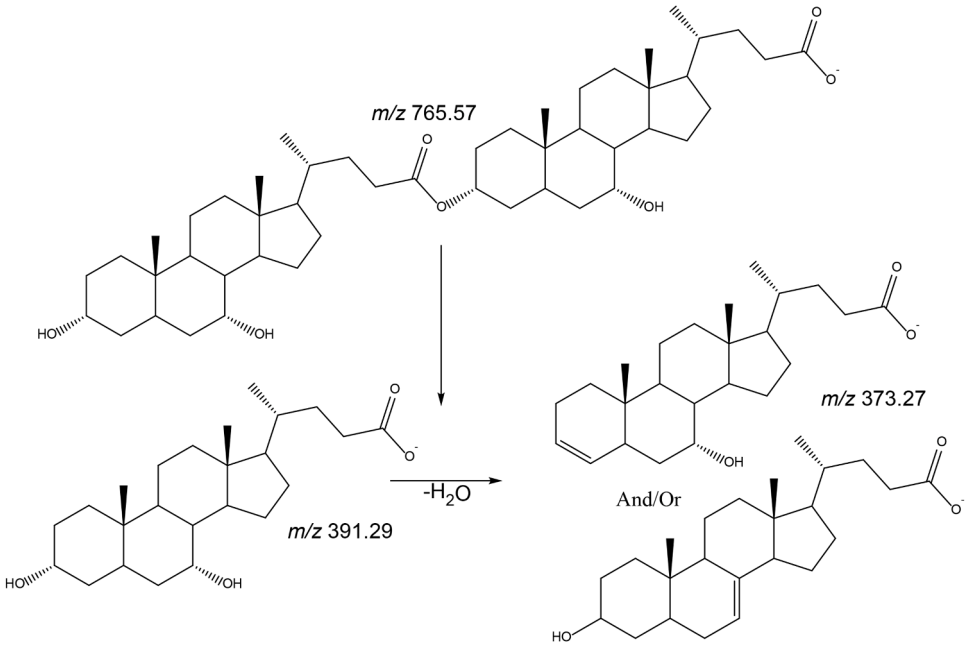



**Fig. 5.** Proposed structure of ester dimer and tandem mass spectrometry fragmentation leading to fragments with mono-isotopic mass of *m/z* 391.29 and *m/z* 373.27.

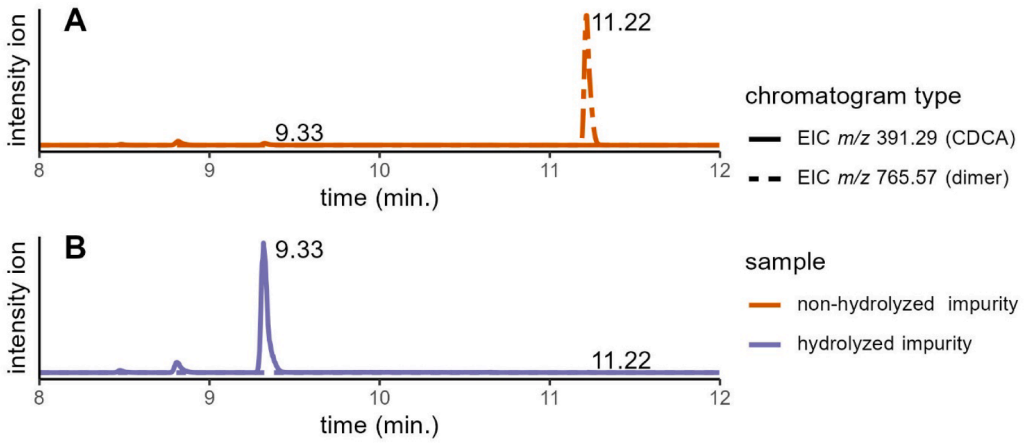



**Fig. 6.** RP-LC-MS/MS before and after alkaline hydrolysis. A: Extracted ion chromatograms (EIC) of *m/z* 391.29 (CDCA) and *m/z* 765.57 (ester dimer) before alkaline hydrolysis. B: EIC of *m/z* 391.29 (CDCA) and *m/z* 765.57 (ester dimer) after alkaline hydrolysis.

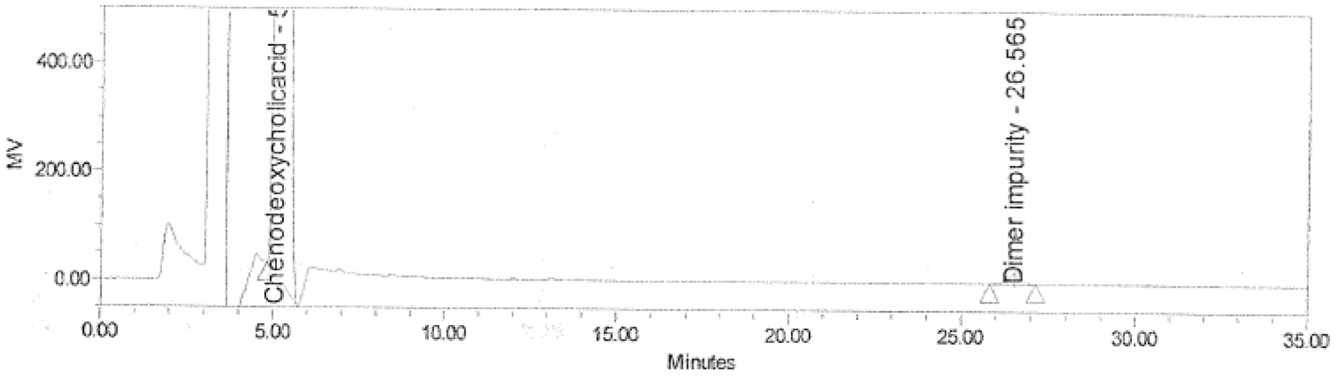



**Fig. 7.** HPLC-RI chromatogram of CDCA API with dimer peak.

**Table 4**
Release QC results of second API.

| Test description | Specification | Results |
|---|---|---|
| Appearance | White or almost white powder | Complies |
| Specific optical rotation | +11.0° to +13.0° | +11.2° |
| Identification (IR) | Positive | Positive |
| Related substances (TLC) | -Ursodeoxycholic acid: Max. 1 % (impurity A) | Complies |
| | -Cholic acid: Max. 0.5 % (impurity B) | Complies |
| | -Lithocholic acid: Max. 0.1 % (impurity C) | Does not comply* |
| | -Any other spot: Max. 0.25 % | Complies |
| | -Two clearly separated principal spots | |
| Related substances (HPLC-RI) | -Impurity I: Max. 0.5 % | 0.3 % |
| Loss on drying | Max. 1.5 % | 0.1 % |
| Sulphated ash | Max. 0.1 % | 0.01 % |
| Assay (dried) | 99.0 % - 101.0 % | 100.7 % |
| Polymorphism | 166 °C ± 3 °C | 168.1 °C |
| Residual Solvents | Complies to Ph.Eur. 5.4 | Complies |
| Microbiology | -TAMC: NMT $10^3$ CFU/g | <5 CFU/g |
| | -TYMC: NMT $10^2$ CFU/g | <5 CFU/g |

* Does not comply due to Imppurity I, which is therefore controlled by HPLC-RI.

the beginning of our investigation that the impurity must be a similar bile acid compound.

In this paper we described the challenges we faced during quality control in our search for a suitable CDCA API for pharmacy compounding of CDCA capsules. In conclusion, we identified and quantified a new impurity in CDCA API which appeared to be a dimer of CDCA. This impurity is abundant in all CDCA APIs, including the API in the commercially available product. The Amsterdam UMC continued pharmacy compounding of CDCA capsules with a CDCA API containing <0.5 % of the dimer impurity. This experience demonstrates that significant time and effort are required to acquire good quality CDCA API, and that sensitive and specific analytical methods are essential for adequate impurity investigation.

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## CRediT authorship contribution statement

**Natalja Bouwhuis:** Writing – review & editing, Writing – original draft, Visualization, Validation, Project administration, Investigation, Formal analysis, Data curation. **Yasmin Polak:** Writing – review & editing, Validation, Project administration, Methodology, Data curation. **Anneliene M. Schimmel:** Writing – review & editing, Validation. **Yuma A. Bijleveld:** Writing – review & editing, Validation. **Martin A. Giera:** Writing – review & editing, Resources, Investigation. **Marieke Heijink:** Writing – review & editing, Resources, Investigation. **Frédéric M. Vaz:** Writing – review & editing, Resources, Investigation. **Albert H. Bootsma:** Writing – review & editing, Resources, Investigation. **Laureen A. ten Berg-Lammers:** Writing – review & editing, Validation. **Noortje E.**

**L. Swart:** Writing – review & editing, Conceptualization. **Carla E.M. Hollak:** Writing – review & editing, Resources, Funding acquisition, Conceptualization. **Bart A.W. Jacobs:** Writing – review & editing. **E. Marleen Kemper:** Writing – review & editing, Validation, Supervision, Resources, Project administration, Methodology, Investigation, Funding acquisition, Formal analysis, Data curation, Conceptualization.

## Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: E. Marleen Kemper & Carla E.M. Hollak report financial support was provided by Nationale Postcode Loterij. Frederic M. Vaz has patent #PCT/EP2018/055,236 issued to Amsterdam UMC. If there are other authors, they declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

## Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.ejps.2024.106979.

## Data availability

Data will be made available on request.

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