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Towards identifying nicomorphine administration in doping control – synthesis of metabolites

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Background: Nicomorphine is rapidly metabolized mainly to the biologically active 6-nicotinoyl morphine and morphine. In sport, morphine and nicomorphine use is prohibited whereas codeine use is permitted. Accredited laboratories routinely test for morphine hence must be able to distinguish morphine, as a metabolite of a prohibited substance, from that whose use is permitted. **Results:** Here we show a relatively simple method to synthesize the nicomorphine metabolites, 3-nicotinoyl- and 6-nicotinoyl morphine, and indicate how they may be used to identify nicomorphine administration. **Conclusion:** This approach should help confirm that it is not codeine, an allowable analgesic in sport, that has been administered.

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1.0 Introduction

Nicomorphine is the 3,6-di-nicotinate ester of morphine with opioid analgesic effects virtually identical to those of morphine but two to three times more potent. It was developed in Europe in the first half of the last century to manage severe pain in

patients with cancer and neuropathic pathologies; its original proprietary name still in use is Vilan®. In some European countries, especially German speaking, nicomorphine is still used for post-operative pain relief. However, nicomorphine currently has no government regulatory approval for human clinical use in the United States and is a Schedule I Controlled Substance under the United States Code (USC) Controlled Substances Act.

Similar to other narcotics, nicomorphine is a powerful painkiller that dulls the nervous system's ability to detect pain, which could allow an athlete to mask pain or injury and reduce stress or anxiety levels. However, it has only been prohibited in sport by World Anti-Doping Agency (WADA) since 2017 and is still included in the 2021 Prohibited List. [101,102]

It is administered orally, by intramuscular, intravenous or subcutaneous routes as well as by rectal administration. When administered by the intramuscular or intravenous routes, nicomorphine is rapidly metabolized to the active metabolite 6-nicotinoyl morphine (6-NM) within 1-3 minutes [1] and then into morphine within about 15 minutes [1-3], as illustrated in **Fig. 1a**. Such rapid serum disappearance could be attributed to both rapid metabolism and rapid tissue distribution of the lipophilic opiate. The final hydrolysis product, morphine, is subsequently metabolized to morphine 3- and 6-glucuronide, which possess half-lives of about 225 minutes [1].

Figure 1 about here

When administered via the rectal route no nicomorphine was detected in serum, whereas morphine appeared almost instantaneously [4]. The two glucuronide forms of morphine were subsequently detected in plasma and urine. Such a pathway was explained by the rapid hydrolysis of nicomorphine into morphine in the colon given its alkaline pH.

Following epidural administration, nicomorphine metabolism is relatively slower [5], a much slower release from the epidural space occurs and nicomorphine and its metabolites remain detectable for about 1.5 h, and have a longer effect of 18.2 ±10.1 h due to slower release of the active metabolites, morphine and 6-NM. Interestingly, none of these studies in humans detected the 3-mononicotinoyl morphine ester (3-NM), suggesting this is not a metabolic intermediate *in-vivo*. However, all the above-mentioned studies relied on HPLC with UV and electrochemical detection and radioimmunoassay [6,7]. Modern sophisticated techniques such as liquid chromatography coupled tandem and/or high-resolution mass spectrometry (LC-MS) could allow a more sensitive detection of nicomorphine or its metabolites in biological matrices such as serum or urine.

Literature searches for information on the oral route of administration were unsuccessful but it is likely that first pass metabolism will quickly convert nicomorphine to 6-NM in a similar way that heroin is converted to 6-monoacetylmorphine.

Given the very short half-life of nicomorphine and its active 6-NM metabolite, it is predicted that WADA accredited laboratories might report adverse analytical findings (AAFs) for the administration of either of those two compounds during doping control and this would be problematic, as the only likely to be detected analytes in urine would be morphine and/or its glucuronide metabolites. This makes the distinction between

morphine or nicomorphine consumption difficult. Although the administered substance does not need to be identified in the urine as part of a doping control test, being able to interpret the analytical findings to determine what has been administered is often needed during the evaluation of a disciplinary case. Furthermore, although WADA has a procedure [103] to help avoid a morphine finding arising from the allowable analgesic codeine being misinterpreted, we consider that the herein reported approach will also minimize the claim that codeine had been administered when, in fact, it was nicomorphine. Here, 3-NM as a synthetic impurity derived from nicomorphine production is demonstrated and a sensitive liquid chromatography-high resolution mass spectrometry (LC-HRMS) detection and identification method for nicomorphine, 3-NM and 6-NM in urine, is presented. This may allow better interpretation of analytical findings related to morphine.

Nicomorphine has been synthesized either via a relatively complex regioselective method to yield the desired isomer [8,9], or via a much simpler non-selective method by acylating morphine with nicotinoyl chloride in the presence of pyridine. [10] Here we exploited a similar approach to this latest relatively simple method of synthesis of nicomorphine, 3-NM and 6-NM. We undertook this work because of difficulty in obtaining these compounds quickly. This route of synthesis is likely to be achievable in most WADA accredited laboratories to help evidence nicomorphine administration.

2.0 Materials and methods

2.1 Chemicals

Nicomorphine standard was obtained from Dr Thomas Geisendorfer from the Austrian WADA accredited Anti-Doping Laboratory in Seibersdorf. Morphine (Cerilliant certified reference material) was obtained from Sigma (Dorset, UK). Dimethylformamide (DMF), tetrahydrofuran (THF), pyridine, 4-dimethylaminopyridine (DMAP), nicotinoyl chloride, formic acid (FA), trifluoroacetic acid (TFA), ammonium hydroxide, sodium sulphate, methanol HPLC grade and acetonitrile HPLC grade were purchased from Fisher Scientific (Loughborough, UK). Ethyl acetate was purchased from Sigma-Aldrich (Dorset, UK). β-glucuronidase from *Escherichia coli* K-12 was purchased from Roche (Basel, Switzerland). Mefruside was purchased from Bayer (Leverkusen, Germany), while d_3-testosterone glucuronide and d_3-salbutamol were purchased from the National Measurement Institute (Sydney, Australia). CP grade oxygen-free nitrogen was purchased from BOC (Guildford, UK). Ultra-pure water (18.2 MΩ.cm) was obtained from an Elga Purelab Flex (High Wycombe, UK).

2.2 NMR

All NMR experiments were performed on a Bruker Avance III HD NanoBay 400 MHz NMR using a 5 mm 1H/13C/15N/31P QNP probe equipped with z-gradient. Two-dimensional correlation spectroscopy (COSY), heteronuclear single quantum coherence spectroscopy (HSQC) NMR spectra were acquired using standard pulse sequences from the Bruker library. All the spectra were acquired in methanol-d4 and the chemical shifts were referenced relative to the residual CHD_2OD signal set at δH 3.31 ppm.

2.3 *Method validation*

Limit of detection (LOD), carry-over and specificity were assessed during method validation. To assess specificity ten different urine samples, from healthy female and male volunteers for use in other validation studies by the WADA accredited laboratory, were spiked with nicomorphine at 25 ng/mL, which is half the minimum required performance level (MRPL) specified by WADA [104]. This was followed by analyzing the same ten different urines that had not been spiked. In order to establish LOD, a single urine sample was spiked at 0.5 ng/mL (1 % MRPL), 5 ng/mL (10 % MRPL) and 25 ng/mL (50 % MRPL). Carryover was investigated by analyzing a spiked urine at 500 ng/mL (10 × MRPL), followed by the non-spiked (blank) urine sample. Furthermore, the stability of nicomorphine was assessed during method validation. Urine aliquots were spiked at 25 ng/mL (50 % MRPL), 50 ng/mL (MRPL) and 100 ng/mL (2 × MRPL) and frozen at -20 °C for one month before the extraction and analysis. Throughout the validation, the analysis of each spiked sample was followed by the analysis of a non-spiked one.

2.4 Sample preparation-solid phase extraction

Sample preparation and analysis was performed following a previously published procedure [11].

Briefly, to each 1 mL spiked urine, the internal standard was added (100 µL of mefruside/$d_3$testosterone glucuronide/d_3-salbutamol (10/1/2 µg/mL, respectively) methanolic solution) followed by 1 mL of *Escherichia coli* β-glucuronidase solution (1:50 v/v) in phosphate buffer (0.1M, pH = 6.2). The hydrolysis was performed for 1 h at 50 ±5 °C using a Heratherm™ incubator (Thermo Fisher Scientific, Hemel Hempstead, UK). After the hydrolysis, 2 mL of 2 % aqueous formic acid solution was

added to the samples and samples were centrifuged at 100 g for 5 min using an IEC Centra GP8 centrifuge (Thermo Fisher Scientific, Hemel Hempstead, UK).

Bond Elute Plexa PCX (Agilent Technologies, Stockport, UK) cartridges (3 mL, 60 mg bed size) were conditioned with 0.5 mL methanol and 0.5 mL 2 % aqueous formic acid solution prior to sample loading. After the urine had passed through, the cartridges were washed with 2 mL of 2 % aqueous formic acid solution, 2 mL water and 2 mL 20 % methanol in water. The elution was performed with a fresh solution of 3 % ammonia in acetonitrile:water (50:50, v/v).

The eluted solvent was evaporated under oxygen-free nitrogen at 60 ±5 °C using a TurboVap® LV (Biotage, Uppsala, Sweden). Samples were reconstituted by adding 100 µL of aqueous solution containing 0.3 % formic acid and 5 % acetonitrile and centrifuged at 850 g for 5 min prior to LC-HRMS analysis.

2.5 LC-HRMS analysis

Liquid chromatography-high resolution mass spectrometry (LC-HRMS) analysis was performed using a Q Exactive™ Hybrid Quadrupole-Orbitrap™ Mass Spectrometer with a HESI II ion source coupled with a Dionex UltiMate™ 3000 UHPLC pump (Thermo Fisher Scientific, Hemel Hempstead, UK). An aqueous and organic mobile phase A (0.3% formic acid in water) and B (0.3 % formic acid in acetonitrile) respectively and Acquity® UPLC BEH C18 column (1.7 µm, 2.1 × 50 mm) (Waters, Elstree, UK) were employed for the separation. The column temperature was thermostated at 30 °C and the autosampler sample compartment chilled to 8 °C. The linear gradient using 0.3 mL/min flow was programmed as: 5 % B (0 min), 20 % B (3.5 min), 25 % B (5.5 min), 57 % B (7 min), 90 % B (8 min) and 5 % B (8.01 to 10 min). The injection volume was 10 µL.

The acquisition was performed in full scan positive ionization mode including all ion fragmentation and full scan negative ionization mode. The resolution was 70,000 FWHM for full scan mode and 35,000 FWHM for all ion fragmentation mode. Data were acquired over the range m/z 100-1000. The AGC target was $1x10^6$ and maximum injection time of 50 ms.

The electrospray source settings were as follows: sheath gas and auxiliary gas flow rates 70 and 10, respectively; spray voltage 3.75 and 3.00 kV for positive and negative ionization, respectively; S-lens RF level 55. The capillary temperature was set at 320 °C; the probe heater temperature was set at 350 °C. Prior to any use, the mass spectrometer was calibrated following the manufacturer's instructions.

Identical chromatographic and MS variables were used for targeted MS2 experiments, where a resolution of 35,000, a precursor isolation width of m/z 1.0 and a NCE = 45 were used.

2.6 Analytical and Preparative HPLC

The semipreparative HPLC was conducted on a Waters Autopurification System using H_2O/0.1 % TFA as eluent A and CH_3CN/0.1 % TFA as eluent B. The elution program started with a linear gradient of 0 % of eluent B to 90 % of B in 60 min. An X-Terra C_{18} 10 x 100 mm semi-preparative column was employed for the chromatographic separation. The detection wavelength was 281 nm. The isolated fractions were further analysed by analytical HPLC/UV on a Hewlett Packard 1050 system (column: Zorbax Extend C_{18}, 5 Å pore size, 2.1 mm x 100 mm; run time 30 min; gradient 0-90 % B over 20 min; A: 0.1 % TFA in water; B: 0.1 % TFA in acetonitrile; flow rate 0.3 mL/min) and data processed with Agilent ChemStation Software (revision A.10.02).

2.7 Chemistry

Synthesis of compound **3-NM**. To a solution of 100 mg of morphine (0.35 mmol) in 5 mL of DMF, was added at 0 °C, 2 eq. (0.7 mmol) of pyridine, 0.1 eq. of DMAP (0.035 mmol) and 1 eq. of nicotinoyl chloride (0.35 mmol). The solution was stirred for 1 h at 0 °C, then overnight at room temperature. The compounds were chromatographed via preparative HPLC/UV using H_2O/0.1 % TFA as eluent A and CH_3CN/0.1 % TFA as eluent B. The elution program started with a linear gradient of 0 % of eluent B to 90 % of B in 60 min. The detection wavelength was 281 nm. ^{1}H NMR (400 MHz, methanol-d_4) δ = 2.20 – 2.40 (m, 2H), 2.87 – 3.22 (m, 6H), 3.36 – 3.51 (m, 2H), 4.19 – 4.34 (m, 2H), 5.03 (d, *J*=7.2, 1H), 5.31 – 5.41 (m, 1H), 5.77 – 5.89 (m, 1H), 6.82 (d, *J*=8.3, 1H), 7.05 (d, *J*=8.3, 1H), 7.67 (ddd, *J*=8.1, 5.0, 0.7, 1H), 8.58 (dt, *J*=8.1, 1.8, 1H), 8.85 (dd, *J*=5.0, 1.8, 1H), 9.29 (s, 1H).

Synthesis of compound **6-NM**. A solution of 100 mg of **nicomorphine** (0.20 mmol) was stirred in H_2O/THF (1:1) 0.5 M HCl at room temperature. The reaction was monitored by HPLC and after 5 h was stopped. The aqueous solution was neutralized with a 0.1 M solution of KOH and the organic compounds were extracted with ethyl acetate. The organic phase was dried with sodium sulphate and the compounds were chromatographed via preparative HPLC/UV as described above. The detection wavelength was 281 nm. ^{1}H NMR (400 MHz, methanol-d_4) δ = 2.08 – 2.18 (m, 1H), 2.25 – 2.36 (m, 1H), 2.91 (dd, *J*=19.8, 6.4, 1H), 2.98 – 3.10 (m, 4H), 3.16 – 3.27 (m, 2H), 3.33 – 3.44 (m, 1H), 4.18 – 4.24 (m, 1H), 5.30 (d, *J*=7.0, 1H), 5.52 – 5.58 (m, 1H), 5.58 – 5.65 (m, 1H), 5.90 – 5.98 (m, 1H), 6.57 – 6.71 (m, 2H), 7.59 – 7.65 (m, 1H), 8.46 – 8.52 (m, 1H), 8.78 (d, *J*=5.0, 1H), 9.16 (s, 1H).

Synthesis of compound **nicomorphine**. To a solution of 100 mg of morphine (0.35 mmol) in 5 mL of DMF, were added at 0 °C, 2 eq. (0.7 mmol) of pyridine, 0.1 eq. of

DMAP (0.035 mmol) and 2.5 eq. of nicotinoyl chloride (0.88 mmol). The solution was stirred over night at room temperature. The compounds were chromatographed via preparative HPLC/UV as described above. ^{1}H NMR (400 MHz, methanol-d_4) δ = 2.22 (d, *J*=14.1, 1H), 2.43 (s, 1H), 2.85 – 3.28 (m, 6H), 3.47 (d, *J*=19.9, 2H), 4.31 (s, 1H), 5.45 (d, *J*=7.2, 1H), 5.55 (dq, *J*=8.1, 2.7, 1H), 5.69 (d, *J*=10.3, 1H), 5.99 (d, *J*=10.3, 1H), 6.90 (d, *J*=8.2, 1H), 7.09 (d, *J*=8.2, 1H), 7.46 (s, 1H), 7.54 (s, 1H), 8.20 (d, *J*=8.0, 1H), 8.33 (d, *J*=8.0, 1H), 8.70 (s, 1H), 8.80 (s, 1H), 8.95 (s, 1H), 9.09 (s, 1H).

3.0 Results and Discussion

Although in theory it is possible to purchase the parent compound and its metabolites, we were unable to find a source for a small quantity at a reasonable cost and delivery time. This caused us to investigate in house synthesis and were able to develop a novel simple and rapid approach.

3.1 Chemistry

Unlike the previously reported synthesis of different 3- and 4- substituted morphine derivatives, where regioselectivity was achieved by the use of specific protecting groups [8,9] , our method exploited the different reactivity of the phenolic and alcoholic -OH groups in positions -3 and -6, respectively. Here, and importantly, the addition of only one equivalent of nicotinoyl-chloride, enables the more reactive phenol group to be selectively functionalized. On the other hand, the hydrolysis of the 3,6-difunctionalized morphine allows the nicotinoyl ester in the phenolic position -3 to be selectively hydrolyzed, leading preferentially to the 6-NM derivative.

Nicomorphine was synthesized from morphine hydrochloride and nicotinoyl-chloride in the presence of catalytic amounts of 4-dimethylaminopyridine (DMAP) in

pyridine/DMF. Besides the presence of nicomorphine, an intermediate with accurate mass at m/z 391.1649 (exact mass m/z 391.1652 for $C_{23}H_{23}N_2O_4^+$) was detected in the LC-HRMS analysis and believed to be 3-NM due to the higher reactivity of the phenol group compared to the alcohol moiety, according to **Fig. 1b**.

LC-HRMS data for the mixture of the detected mono-nicotinoyl-morphine ester intermediate (finally assigned as 3-NM) and nicomorphine are presented in **Fig. 2**:

Figure 2 about here

A peak at 4.25 min was detected as the main species that was considered to be the final product, nicomorphine (accurate mass m/z 496.1866, exact mass m/z 496.1867 for $C_{29}H_{26}N_3O_5^+$ and accurate mass m/z 248.5969, exact mass m/z 248.5970 for $C_{29}H_{27}N_3O_5^{2+}$), alongside residual morphine (0.9 min) and a peak at 3.16 min with accurate mass m/z 391.1647 (exact mass m/z 391.1652 for $C_{23}H_{23}N_2O_4^+$). The latter was attributed to 3-NM, which is a potential metabolite and/or by product never detected in previous studies. Although 3- and 6-NM are of identical elemental compositions, a baseline separation of the two isomers is achievable with a suitable UHPLC system. Thus, it was decided to investigate further the possibility of distinguishing 3- from 6-NM.

To help identify the species eluting at 2.99 min, pure nicomorphine was isolated from the crude synthetic mixture via liquid-liquid extraction (LLE) followed by

semi-preparative-HPLC-DAD-MS, dissolved in 95 % aqueous acetonitrile containing 0.3 % FA and incubated for 5 days to allow mild acidic hydrolysis at the phenolic position. This was followed by LC-HMRS analysis, presented in **Fig. 3**:

Figure 3 about here

As expected, mild acidic hydrolysis produced selectively the 6-NM isomer (RT = 2.98 min in **Fig. 3**) due to the much more labile nature of the phenolic ester. Therefore, it was preliminary considered that the species detected at 2.98 min was attributable to the 6-NM, whereas the species eluting at 3.16 min could be attributed to 3-NM.

Nicomorphine and the two separated isomers were subsequently subjected to LC-MS/HRMS; the collision energy used for the MS/MS fragmentation of the precursor ions was identical for all the three analytes. The product ion spectra and possible structures of fragments ions are shown in **Figs. S1–S3** (supplementary information).

Fragmentation of nicomorphine was characterized by the loss of a single nicotinic acid group, presumably at the labile phenolic region, alongside the formation of the N-ethylidene methanaminium ion at m/z 58.0654 (exact mass m/z 58.0651 for $C_3H_8N^+$). The other main characteristic product ions were at m/z 106.0287 (exact mass m/z 106.0287 for $C_6H_4NO^+$) and m/z 78.0338 (exact mass m/z 78.0338 for $C_5H_4N^+$), both deriving from further fragmentation of the nicotinic acid moiety. See **Table 1** for the relevant accurate masses, the equivalent elemental composition and exact mass.

Interestingly, product ion spectra of the two mononicotinic derivatives, namely 3- and 6-NM, were somewhat different both in product ion composition and product ion ratios.

Both isomers underwent nicotinic acid loss, alongside formation of the N-ethylidene methanaminium ion. However, in 3-NM, a product ion was observed at m/z 373.1544 (exact mass m/z 373.1547, for $C_{23}H_{21}N_2O_3^+$) due to water loss, which was not a favored fragmentation in the 6-NM derivative. This was attributed to the more favored electron rearrangement in the aliphatic ring as a consequence of water loss from the alcoholic position in the 3-nicotinoyl morphine analogue. 6-NM also underwent fragmentation such that only a few product ions derived from the polycyclic morphine scaffold [12-15] were distinguishable at relatively high abundances, such as ions observed at m/z 211.0753 (exact mass m/z 211.0754 for $C_{14}H_{11}O_2^+$), m/z 193.0649 (exact mass m/z 193.0648 for $C_{14}H_9O^+$) and m/z 165.0699 (exact mass m/z 165.0699 for $C_{13}H_9^+$). Such scenario could again be attributed by retention of the phenolic group in 6-NM. Indeed, the ion at m/z 106.0287 (exact mass m/z 106.0287 for $C_6H_4NO^+$), attributed to the nicotinic acid fragment rearrangement, was much more abundant and dominant in 3-NM, reflected by the labile phenolic ester nature when compared to the aliphatic ester moiety. In summary, targeted MS/MS and UHPLC-HRMS was a valuable tool to detect and identify nicomorphine and its hydrolysed metabolite species.

3.2 Purification and NMR analysis of nicomorphine derivatives

To confirm the correct mononicotinoyl isomers assignment undertaken via LC-HRMS, nicomorphine, 3-NM and 6-NM were isolated in pure form and fully characterized via HPLC-DAD, LC-HRMS as well as via 1D and 2D ^{1}H NMR. Following synthesis of nicomorphine, 3-NM and 6-NM (**Fig. 1**), the compounds were chromatographed via semi-preparative HPLC-DAD-MS. The analytical chromatograms (HPLC-DAD on a

C_{18} column) of the resulting isolated pure analytes are reported in **Fig. S4** (supplementary information).

While pure fractions were analyzed via UHPLC-MS/HRMS, the structures of nicomorphine, 3-NM and 6-NM were also further elucidated via NMR spectroscopy. Initially, NMR investigation with the assignment and quantification of ^{1}H signals of morphine in CH_3OD was undertaken, and nicomorphine, 3-NM and 6-NM were then compared to the reference spectra of morphine. Attention was focused on the protons in positions 5–9 (**Table 2**). After in-depth analysis of the 1D^{1}H (**Fig. S5**, supplementary information), 2D COSY (**Fig. S6**, supplementary information) and 2D HSQC (**Fig. S7,** supplementary information) spectra the signals of the morphine protons were assigned as follows: δ 5.77 (ddt, J = 9.9, 3.2, 1.5 Hz, 1H, H_7), 5.33 (dt, J = 9.9, 2.7 Hz, 1H, H_8), 4.93 (dd, J = 6.3, 1.2 Hz, 1H, H_5), 4.26 (dq, J = 5.6, 2.6 Hz, 1H, H_6), 4.17 (dd, J = 7.0, 3.2 Hz, 1H, H_9). The two-dimensional spectrum that results from the COSY experiment showed that the H_9 have cross peaks with the aliphatic proton at δ 2.90, presumably $H_{10a\text{-}b}$, and the ^{1}H-^{13}C HSQC experiment showed a coupled carbon at 61.01 ppm. The H_6, δ 4.26, showed COSY cross peaks with H_5, H_7 and H_8, and is coupled to a carbon at 66.08 ppm. A carbon at 90.45 ppm, which was assigned to the carbon at position 5, is bound to a proton at 4.93 ppm, H_5, and the COSY spectra revealed cross peaks with H_6 and H_7. The other two signals, 5.77 and 5.33 ppm, are clearly the ones of the protons H_7 and H_8, respectively. H_7 have cross peaks with H_6, H_5 and H_8 and were bound to a carbon at 134.30 ppm. H_8 have cross peaks with H_6 and H_7 and were bound

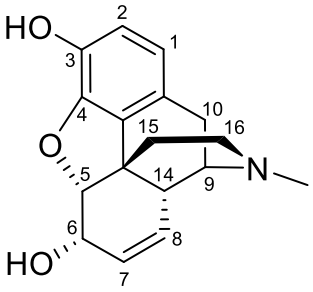
to a carbon at 124.55 ppm. Once the protons of morphine in CH_3OD were assigned, the NMR spectra of nicomorphine, 3-NM and 6-NM were compared with the spectra of that of morphine and analysed (**Fig. S13,** supplementary information). The structure of 3-NM and 6-NM were determined by ^1H NMR comparison. As expected, the structures of the two derivatives were confirmed by the chemical shift of the protons at position 6 (**Fig. S13**, supplementary information). The chemical shift of this proton is not influenced by 3-NM; however it changes significantly when the nicotinic moiety is attached in position 6 as well as in nicomorphine. The H_6 chemical shift remains at around 4.26 ppm in 3-NM, however it shifts to 5.55 and 5.56 ppm in nicomorphine and 6-NM, respectively. The observed shift was attributed to the presence of a substituent in position 6, in both nicomorphine and 3-NM. The COSY spectrum of the nicomorphine clearly demonstrates the correct chemical assignment: the H_6 at 5.55 ppm shows cross peaks with three protons at: 5.45, 5.69 and 5.99 ppm, H_5, H_8 and H_7 respectively, **Fig. S8** (supplementary information). The complete and integrated ^1H NMR of morphine, nicomorphine, 3-NM and 6-NM are reported in **Figs. S9-S12** (supplementary information).

Table 1. Accurate mass of protonated nicomorphine, 3- and 6-nicomorphine and their protonated fragment ions together with their elemental composition, exact mass and mass error

Accurate mass (m/z)	Elemental composition	Exact mass (m/z)	Mass error (ppm)
nicomorphine			
496.1865	$C_{29}H_{26}N_3O_5^+$	496.1867	-0.4
373.1543	$C_{23}H_{21}N_2O_3^+$	373.1547	-1.1
124.0393	$C_6H_6NO_2^+$	124.0393	0.0
106.0287	$C_6H_4NO^+$	106.0287	0.0
78.0338	$C_5H_4N^+$	78.0338	0.0
58.0654	$C_3H_8N^+$	58.0651	5.2

6-nicomorphine			
391.1651	$C_{23}H_{23}N_2O_4^+$	391.1652	-0.3
268.1332	$C_{17}H_{18}NO_2^+$	268.1332	0.0
211.0753	$C_{14}H_{11}O_2^+$	211.0754	-0.5
193.0649	$C_{14}H_9O^+$	193.0648	0.5
165.0699	$C_{13}H_9^+$	165.0699	0.0
124.0393	$C_6H_6NO_2^+$	124.0393	0.0
106.0287	$C_6H_4NO^+$	106.0287	0.0
78.0339	$C_5H_4N^+$	78.0338	1.3
58.0654	$C_3H_8N^+$	58.0651	5.2
3-nicomorphine			
391.1649	$C_{23}H_{23}N_2O_4^+$	391.1652	-0.8
373.1544	$C_{23}H_{21}N_2O_3^+$	373.1547	-0.8
250.1225	$C_{17}H_{16}NO^+$	250.1226	-0.4
124.0393	$C_6H_6NO_2^+$	124.0393	0.0
106.0287	$C_6H_4NO^+$	106.0287	0.0
78.0339	$C_5H_4N^+$	78.0338	1.3
58.0654	$C_3H_8N^+$	58.0651	5.2

Table 2. Structure of morphine, chemical shifts of H_1-H_9 for morphine, and compounds **1**-**3**.



Position	Morphine			3-NM			6-NM			nicomorphine		
	δ (ppm)	Multiplicity	*J* (Hz)	δ (ppm)	Multiplicity	*J* (Hz)	δ (ppm)	Multiplicity	*J* (Hz)	δ (ppm)	Multiplicity	*J* (Hz)
1	6.57	D	8.2	6.82	D	8.3	6.64	m		6.90	d	8.2
2	6.64	D	8.2	7.05	D	8.3	6.64	m		7.09	d	8.2
5	4.93	Dd	6.3, 1.2	5.03	D	7.2	5.30	d	7.0	5.45	d	7.2
6	4.26	Dq	5.6, 2.6	4.28	M		5.56	m		5.55	dq	8.1, 2.7
7	5.77	Ddt	9.9, 3.2, 1.5	5.83	M		5.94	m		5.99	d	10.3
8	5.33	Dt	9.9, 2.7	5.35	M		5.62	m		5.69	d	10.3
9	4.17	Dd	7	4.25	M		4.21	m		4.31	s	

3.3 Validation results

A summary of the validation results is shown in **Table 3.**

Table 3. Summary of validation results.

Limit of detection	Dilutions down to 1 % of MRPL (0.5 ng/mL) were analyzed. (see **Figs. S14 & S15,** (supplementary information))	A clear peak was still detectable at 5 ng/mL but not at 0.5 ng/mL.
Stability	Nicomorphine peak was detected at 50 % MRPL, MRPL and 2 × MRPL after spiked samples stored for 1 month were extracted and analysed.	
Carryover	Nicomorphine at 10 × MRPL was analyzed by LC-HRMS.	No nicomorphine peak greater than 0.1 % was detected in the negative control sample next analyzed.
Specificity	Nicomorphine peak was detected at 50 % MRPL in all spiked ten urine samples.	No interfering peak was detected in any of the blank urine samples.

No suitable standard was available for either 3-NM or 6-NM to be able to determine their LODs.

Previous research in the area demonstrated that 3-NM was undetectable in biological matrices [1-5,16]. However, analysis was performed via HPLC in combination with UV and electrochemical detection. It is reasonable to hypothesize that modern high-resolution LC-MS or tandem MS/MS instruments will provide superior sensitivity and thus the ability to detect both 3-NM and 6-NM.

Indeed, an authentic nicomorphine standard was spiked in urine at 50 ng/mL, which is the current WADA minimum required performance level (MRPL) [104] for all narcotics (apart from buprenorphine (5 ng/mL MRPL) and fentanyl and derivatives (2 ng/mL MRPL)) and, following solid-phase extraction (SPE), the sample was analysed via UHPLC-HRMS. The data, shown in **Fig 4.**, demonstrate detection of the expected nicomorphine peak at 4.25 min. alongside a peak at m/z 391.1642 but at 2.99 min, which can be attributed to 6-NM. Furthermore, a minor peak at 3.16 min due to 3-NM

was observed. No residual morphine was detected in this sample. This clearly indicates the possibility of distinguishing and potentially detecting nicomorphine or its partially hydrolysed species in urine samples.

Figure 4 about here

Serial dilution of the spiked urine sample with blank urine led to an estimated limit of detection (LOD) of 5 ng/mL using LC-HRMS. Presumably, targeted LC-MS/MS on modern triple quadrupole mass spectrometers will lead to even lower LODs for such analytes, demonstrating the potential of detecting extremely low level of synthetic or naturally derived nicomorphine intermediates and metabolites, respectively.

4.0 Conclusion & Future Perspective

In this work, nicomorphine, 6-NM and 3-NM were synthesized and characterised via NMR and LC-HRMS and LC-MS/HRMS. A UHPLC method was developed to separate the three analytes and a urine extraction method demonstrated the ability to detect nicomorphine at or below the WADA MRPL. Given the extremely short half-life of nicomorphine, 6-NM especially could represent a novel analytical marker for the detection of nicomorphine misuse. It is hoped that both the simple synthetic chemistry procedure for the preparation of nicomorphine and its metabolites, and the developed analytical method, will be useful for anti-doping laboratories wishing to include these morphine-derivatives in their screening and confirmatory analytical procedures.

Financial & competing interests disclosure

The authors have no relevant affiliations or financial involvement with any organization or entity with a financial interest in or financial conflict with the subject matter or materials discussed in the manuscript. This includes employment, consultancies, honoraria, stock ownership or options, expert testimony, grants or patents received or pending, or royalties.

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Executive summary

- Nicomorphine is rapidly metabolized to morphine and hence is indistinguishable from the latter and both are prohibited in sport
- Codeine, which is permitted in sport, is also metabolized to morphine
- The intermediary nicomorphine metabolite 3- and 6-nicomorphine may be readily synthesized and especially 6-nicomorphine could be used to assist in detecting nicomorphine administration

Reference annotations

* reference 1. Koopman-Kimenai PM, Vree TB, Booij LHDJ, Dirksen R. Pharmacokinetics of Intravenously Administered Nicomorphine and its Metabolites and Glucuronide Conjugates in Surgical Patients. *Drug Investigation*, 7(2), 63-73 (1994)

This publication is a useful source of the pharmacokinetics data of nicomorphine and its metabolites after intravenous administration.

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This publication shows the mass spectrometric fragments of codeine and morphine that is relevant for comparison with those of nicomorphine and its metabolites.

5.0 References

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List of Figure Captions

Figure 1: a) Proposed nicomorphine metabolism pathway. b) Synthesis of nicomorphine and hydrolysis of nicomorphine to 6-NM

Figure 2: Analysis of synthetic nicomorphine intermediate mixture. Top panel: extracted ion chromatograms of morphine (0.90 min), mono-nicotinoyl-morphine - finally assigned as 3-NM (3.16 min) and nicomorphine (4.25 min), respectively. Middle panel: HRMS spectrum of mono-nicotinoyl-morphine (finally assigned as 3-NM). Bottom panel: HRMS spectrum of nicomorphine.

Figure 3: Analysis of chemically-hydrolyzed nicomorphine. Top panel: extracted ion chromatograms of morphine (0.90 min), mono-nicotinoyl-morphine - finally assigned as 6-NM (2.98 min) and nicomorphine (4.26 min), respectively. Bottom panel: HRMS spectrum of mono-nicotinoyl-morphine (finally assigned as 6-NM).

Figure 4: Analysis of a urine sample spiked with nicomorphine: extracted ion chromatograms for mono-nicotinoyl-morphine (3- and 6- nicotinoyl morphine) (top panel) and nicomorphine (bottom panel).