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Abstract:	

The first reported fatality associated with the synthetic opioid 3,4-dichloro-*N*-[2-(dimethylamino)cyclohexyl]-*N*-methylbenzamide (U-47700) and implications for forensic analysis

Simon P. Elliott, [a,*] Simon D. Brandt [b], Christopher Smith [a]

[a] *ROAR Forensics, Malvern Hills Science Park, Geraldine Road, Malvern WR14 3SZ, UK*

[b] *School of Pharmacy and Biomolecular Sciences, Liverpool John Moores University, Byrom Street, Liverpool L3 3AF, UK*

* Correspondence to: Dr. Simon Elliott, ROAR Forensics, Malvern Hills Science Park, Geraldine Road, Malvern, Worcestershire, WR14 3SZ, UK; E-mail: simontox@yahoo.co.uk

Running title: First reported fatality involving the synthetic opioid U-47700

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Introduction

The search for synthetic opioids as alternatives to opium-based derivatives has provided an important impulse to drug development around the globe. An important goal in the systematic evaluation of new drug candidates is the identification of compounds that provide a more favorable side-effect profile, which includes reduced dependence-producing properties and abuse liability. A rich source of information about these research efforts can be found in the scientific literature. However, the exploration of these important discoveries has also been increasingly mined by large-scale producers of these materials, which are then offered for sale. These so-called 'research chemicals' or new psychoactive substances (NPS)[1] have created challenges to policy makers, clinicians, and law enforcement around the world.[2]

Recent examples of synthetic opioids that emerged as NPS on the market, and which were associated with severe cases of adverse effects, include 3,4-dichloro-*N*-{[1-(dimethylamino)cyclohexyl]methyl}benzamide (AH-7921), 1-cyclohexyl-4-(1,2-diphenylethyl)piperazines (MT-45) and *N*-phenyl-*N*-[1-(2-phenylethyl)piperidin-4-yl]acetamide (acetylfentanyl), respectively (Figure 1). Following the recommendation provided by the World Health Organization's Expert Committee on Drug Dependence (ECDD),[3] AH-7921 was placed in Schedule I of the 1961 Single Convention, as amended by the 1972 Protocol in 2015.[4] Furthermore, ECDD's recommendation to place MT-45 into Schedule I and acetylfentanyl in Schedules I and IV of the same Convention[5] have been recently confirmed by the Commission on Narcotic Drugs.[6]

3,4-Dichloro-*N*-[2-(dimethylamino)cyclohexyl]-*N*-methylbenzamide (U-47700) (Figure 1) has recently emerged on the market and can be purchased from various Internet retailers and is a structural isomer of AH-7921 (Figure 1). The preparation of U-47700 and other derivatives was disclosed by the Upjohn Company in the 1970s[7] followed by the recognition that U-47700 showed increased analgesic properties and morphine-like behavioural features in mice compared to morphine itself.[8,9] The presence of two chiral centres gives rise to a *cis*- and *trans*- racemic mixture with the *trans*-form being advertised for sale. Binding studies also revealed that U-47700 displayed an appreciable selectivity for the μ-opioid receptor over the κ–opioid receptor.[10,11] A variety of cyclohexyl *trans*-1,2-diamines have been found to be potent analgesics and the vicinal 1,2-diamine pattern has provided access to a large range of substances with diverse biological activities.[12-14]

Since U-47700 did not progress to clinical trials, there is no direct clinical information pertaining to its effects. Keeping in mind the various limitations that may be associated with descriptions obtained from self-reporting users, its effects have been described with various positive and negative symptoms but appeared to be essentially comparable to other opioids. Specifically, euphoria was reported in individuals, sometimes being short-lived, as well as general lift in mood with these desired effects being experienced in waves. The negative effects were also opioid-based, including nausea with some users describing respiratory depression. For some users, U-47700 had a shorter duration of action and the urge to keep re-dosing was stated as being very high.[15,16]

Case History

In January 2016, a 27-year old male was found dead at home. It was believed he may have snorted mirtazapine (prescribed anti-depressant) and was a user of cannabis, ketamine, “MCAT” (cathinones) and “legal highs”. There was evidence of powder in his nasal area, which was obtained for analysis. At autopsy, no natural disease or cause of death was found. At the direction of HM Coroner, toxicological analysis was performed for alcohol and a wide range of drugs.

Experimental

Reagents and standards

All solvents and chemicals used, e.g. acetonitrile, 1-chlorobutane, sodium carbonate, sulphuric acid, formic acid, triethylammonium phosphate buffer (TEAP) and ammonium formate, were of analytical grade or equivalent from Sigma Aldrich (Dorset, UK) and/or Rathburn Chemicals Ltd. (Walkerburn, Scotland, UK). A sample of *trans*-U-47700 was obtained from Scientific Supplies Ltd. (London, UK).

Instrumentation

HPLC-DAD analysis was performed using a Dionex 3000 Ultimate liquid chromatography system coupled to a UV diode array detector (Thermo Fisher, St

Albans, UK). An ABSciex 3200 QTRAP mass spectrometer coupled to an Agilent 1200 HPLC-DAD system (ABSciex, Cheshire, UK) was used for LC-MS/MS analysis. An Agilent UHPLC-high resolution QTOF-MS system was used incorporating an Agilent 6540 UHD Accurate-Mass QTOF LC/MS coupled to an Agilent 1290 Infinity UHPLC system (Agilent, Cheshire, UK). The methodology and instrument parameters have been published previously.[17]

Preparation of standards and method characterization

U-47700 was used to prepare fresh reference and calibration standards for the formal identification and quantitation in the specimens analyzed. Following determination of limit of detection (LOD) using an extended calibration range (from 0.025 and 0.078 mg/L) and limit of quantitation (LOQ), a calibration range of 0.3125, 0.625, 1.25, 2.5 and 5 mg/L was produced for U-47700 using blank equine plasma for validation according to Peters *et al*.[18] Internal quality control standards of 0.5 mg/L and 2.5 mg/L were also produced. Intra-day and inter-day precision and accuracy were determined. For quantification, the post-mortem blood case sample was diluted 3-fold in equine plasma for matrix matching and replicate analysis.

Extraction and analysis

Basic back extraction using sodium carbonate buffer (with internal standards) and 1-chlorobutane solvent extraction of the calibration and case samples (blood and urine) was performed as previously described.[17] The chromatographic conditions for qualitative HPLC-DAD, HPLC-MS and UHPLC-QTOF-MS analysis were also based on previously published methods involving an acetonitrile gradient.[17] Quantitative HPLC-DAD analysis was based on 30% acetonitrile (with 25 mM TEAP buffer) under isocratic elution conditions at a flow rate of 2 mL/min. U-47700 eluted at 2.4 min.

Results and discussion

Routine toxicological analysis of the post-mortem urine detected quetiapine (an antipsychotic), amphetamine (a psychostimulant), amitriptyline (an antidepressant), mexedrone (a new psychoactive substance) and ketamine (a dissociative anesthetic). Quetiapine (< 0.05 mg/L) and amphetamine (< 0.1 mg/L) were also detected in the post-mortem blood along with naproxen (an anti-inflammatory drug < 0.8 mg/L) representing therapeutic (or recreational in the case of amphetamine) concentrations. No ethanol (alcohol) was detected. Aside from these findings, during HPLC-DAD analysis, initially unidentified compounds were detected in the blood and urine.

Differentiation between U-47700 and AH-7921

Subsequent analysis by targeted LC-MS (applied to identify a range of specific NPS) and non-targeted QTOF-MS, originally suggested the primary compound to be consistent with AH-7921 (Figure 1) based on ion transitions *m/z* 329 > 173 and *m/z* 329 > 284, with only a very slight difference in retention time. The accurate mass of

the protonated molecule at *m/z* 329.11812 was also consistent with $C_{16}H_{23}Cl_2N_2O^+$. However, both the retention time and UV spectrum differed when analyzed by the HPLC-DAD method. The primary compound with the identical analytical features was also found to be the constituent in the powder recovered from the deceased's nostrils.

Given knowledge of the existence of U-47700 on the NPS market, along with its structural similarity to AH-7921, U-47700 reference material was analyzed using HPLC-DAD, LC-MS and LC-QTOF-MS. Analysis confirmed the primary compound detected in the powder, post-mortem blood and urine to be U-47700 instead of AH-7921. Specifically, under HPLC-DAD conditions, U-47700 eluted at 7.85 min with a maximum peak at 201.7 nm compared to AH-7921 that eluted at 8.26 min with peaks at 205.4 nm and 241.3 nm. Figure 2 shows the overlaid UV spectra and the chromatographic separation of U-47700 from AH-7921. The separate spectra are provided as Supporting Information.

Analysis by triple quadrupole/linear ion trap LC-MS showed that both U-47700 and AH-7921 shared some primary product ions, such as *m/z* 145, 173 and 284, which was in agreement with mass spectral data reported previously.[17,19,20] However, the product ions of interest, which allowed for the differentiation of both isomers, were detected at *m/z* 204 and *m/z* 81 (U-47700) and *m/z* 190 and *m/z* 95 (AH-7921), respectively (Figure 3). For example, in the case of AH-7921, the *m/z* 190 ion would have been consistent with the protonated 3,4-dichlorobenzamide product ion whereas *m/z* 204 reflected the 3,4-dichloro-*N*-methylbenzamide counterpart found in U-47700 (Figure 1). Correspondingly, the differences between *m/z* 81 (U-47700) and *m/z* 95 (AH-7921) reflected the presence of the methylene group in AH-7921 that gave rise to a cyclohex-1-en-1-ylmethylium ion, thus, representing the mass shift of 14 amu. It is therefore recommended that if solely relying on a targeted ion transition methodology for the detection of U-47700, the transitions *m/z* 329 > 81 and *m/z* 329 > 204 will provide the appropriate specificity. Equally, specific transitions *m/z* 329 > 95 and *m/z* 329 > 190 should be incorporated into any LC-MS method that aims to target the detection of AH-7921.

Analysis of femoral blood by high accuracy mass spectrometry confirmed the protonated molecule of U-47700 at *m/z* 329.11812 compared to the reference standard at *m/z* 329.11815 (Figure 4A and B). Both values were within 0.5 ppm of the calculated value at *m/z* 329.11820. The chlorine related isotopic masses linked to the two chlorine atoms (M+2 and M+4) were also evident (Figure 4A). A representative example of a collision-induced dissociation tandem mass spectrum obtained from the analyte peak of a urine sample is shown in Figure 4C. The specific accurate mass product ions at *m/z* 81.07023 ($C_6H_9^+$, Δ = 4.32 ppm) and 203.99811 ($C_8H_8Cl_2NO^+$, Δ = 1.76 ppm) were consistent with the differentiating ions mentioned above (Figure 3). The suggested structures for these product ions are shown in Figure 4G.

Detection of metabolites

During the analysis of the case samples, apparent metabolites of U-47700 were also

detected, with greater abundance in blood compared to urine. Initially, this was observed by HPLC-DAD analysis through UV spectral comparison with the parent molecule and further confirmation was obtained from LC-MS investigations, which pointed towards similar product ions to U-47700 recorded under full scan mass spectrometry conditions. When high accuracy QTOF-MS analysis was carried out, the associated empirical formulae could be determined (Figure 4D-F) which pointed toward *N*-desmethyl and *N*,*N*-didesmethyl products. The appearance of AH-7921 *N*-desmethyl metabolites was consistent with those observed in the authors' laboratory and those reported in the literature.[17,19,20] The detection of the *m/z* 204 ion, i.e. protonated 3,4-dichloro-*N*-methylbenzamide (Figure 4G and Supporting Information), suggested that *N*-desmethylation must have occurred on the *N*,*N*-dimethylcyclohexanamine moiety (Figure 4H). Involvement of the *N*-methyl group would have otherwise given rise to the *m/z* 190 species and this was not observed under the conditions used. QTOF-MS/MS data for *N*-demethyl-U47700 identified in blood are provided as Supporting Information. The detection of the *N*,*N*-didesmethyl-U-47700 metabolite was based on QTOF-MS alone (Figure 4F) but the signal intensity was not sufficient to obtain acceptable QTOF-MS/MS data. The exact nature of this metabolite remained to be confirmed. The suggested structure (Figure 4H) represented the primary amine species, i.e. carrying the *N*-(2-aminocyclohexyl) moiety. However, in the absence of high accuracy MS/MS data, this identification must remain speculative. Similar to CID-MS/MS data recorded for the *N*-desmethyl-U-47700 metabolite (Figure 4D and G), a key product ion that might be expected for the primary amine metabolite would have been at *m/z* 203.99775.

Quantitative analysis

For quantitative analysis by HPLC-DAD, validation of the method showed intra-day accuracy and precision values of < 2% (at 0.5 and 2.5 mg/L), inter-day accuracy and precision values of < 18% and < 6%, respectively (at 0.5 and 2.5 mg/L), a limit of detection of 0.05 mg/L and a limit of quantitation of 0.3125 mg/L utilizing the lowest calibrator. U-47700 was subsequently measured at a concentration of 1.46 mg/L in post-mortem femoral blood.

As there are currently no other published fatalities involving U-47700, concentration data obtained from post-mortem blood cannot be used for comparison. However, if related to morphine or AH-7921 blood concentrations, a femoral blood concentration of 1.46 mg/L could be considered excessive. During the investigation of AH-7921 deaths by this laboratory, post-mortem femoral blood AH-7921 concentrations of 0.05, 0.35, 0.58, 0.84 and 4.46 mg/L were found. Other drugs and/or alcohol were detected in all of the cases but only contributed or provided an alternative cause of death in two of the cases (associated with femoral blood concentrations of 0.05 and 0.35 mg/L). Other deaths in Europe reported by various researchers involved AH-7921 concentrations between 0.03 and 0.99 mg/L.

Nevertheless, as with all fatalities involving opiates and opioids, the toxicological significance will depend on the degree of any acquired tolerance through regular use. In the case described here, there was no information or evidence of regular opiate/opioid use and it was not clear whether the deceased would have known that

the powder used was an opioid, irrespective of the exact composition (i.e. U-47700). Even if it were known to be an opioid, the purity of the substance would have most likely been unknown to the user, creating a dose-safety risk. As nasal insufflation of synthetic opioids (i.e. AH-7921) has been previously reported (and is also commonly associated with other drugs especially cocaine and synthetic stimulants), the route of administration in this case did not provide an inference of user knowledge as to the nature of the powder. Nonetheless, the major risk to life from opioids is their depressant effect on the central nervous system, notably causing respiratory depression, and in the absence of any other significant pathological or toxicological findings, fatal U-47700 toxicity was a likely outcome.

Conclusion

The new psychoactive substance U-47700, a synthetic μ-opioid receptor agonist and related to AH-7921, has recently emerged on the "research chemical" market. The fatality identified in this case presents for the first time a post-mortem femoral blood concentration, namely 1.46 mg/mL, and characterized metabolites. The combination of HPLC-DAD analysis with triple quadrupole/linear ion trap mass spectrometry (MS) and high accuracy QTOF-MS/MS allowed for an unambiguous identification. Given that U-47700 is a structural isomer of AH-7921, another synthetic opioid that has emerged in previous years, care has to be taken if relying solely on accurate mass (without fragmentation) and when choosing ion transitions for targeted analysis, in order to avoid misidentification.

Acknowledgement

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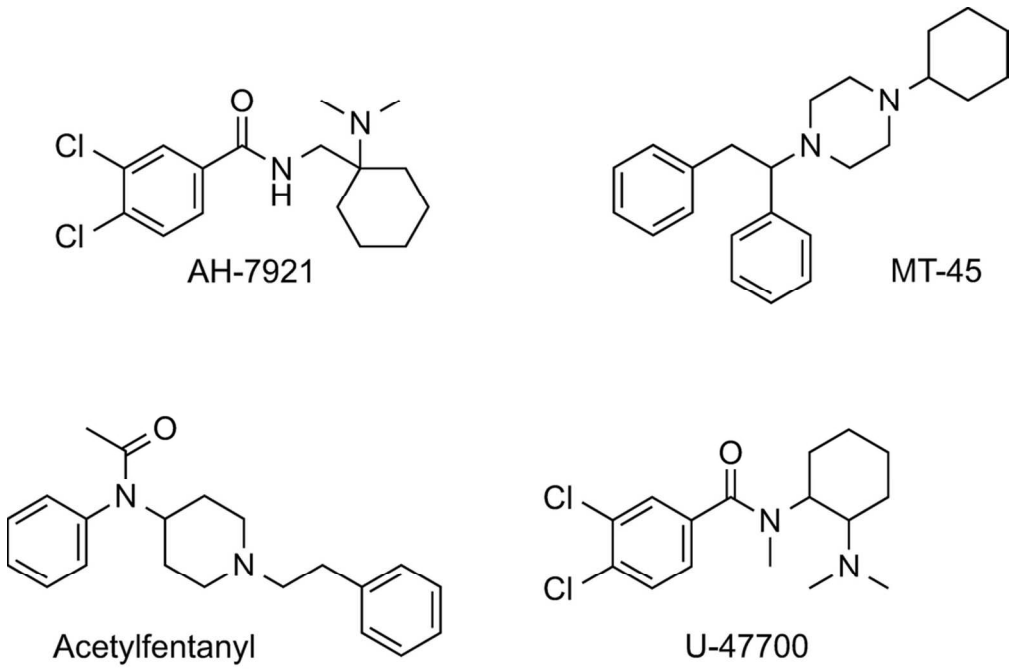
Figure captions

Figure 1. Examples of synthetic opioids that have emerged on the "research chemical" market. U-47700 has only emerged very recently but AH-7921, MT-45 and acetylfentanyl have been placed under international control.

Figure 2. High performance liquid chromatography photodiode array detection data obtained from U-47700 and AH-7921 standards.

Figure 3. Enhanced product ion scans of U-47700 and AH-7921 using electrospray ionization HPLC linear ion trap mass spectrometry. Both structural isomers were differentiated based on distinct product ions as indicated by the arrows.

Figure 4. A–F: UHPLC high accuracy QTOF-MS and MS/MS data recorded for U-47700 and metabolites detected in post-mortem blood and urine. H: Structures of suggested CID-MS/MS key ions recorded for U-47700 and *N*-desmethyl-U-47700. These two key ions would not be detected in the tandem mass spectrum of its structural isomer AH-7921. H: Suggested structures for *N*-desmethyl-U-47700 and *N,N*-didesmethyl-U-47700.



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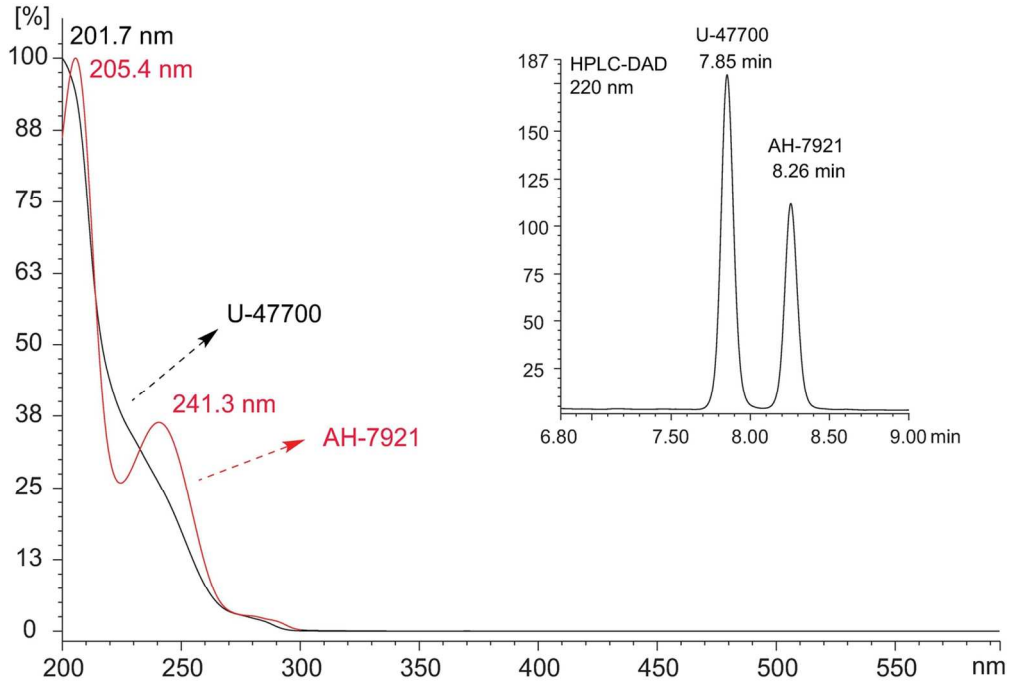

High performance liquid chromatography photodiode array detection data obtained from U-47700 and AH-7921 standards.
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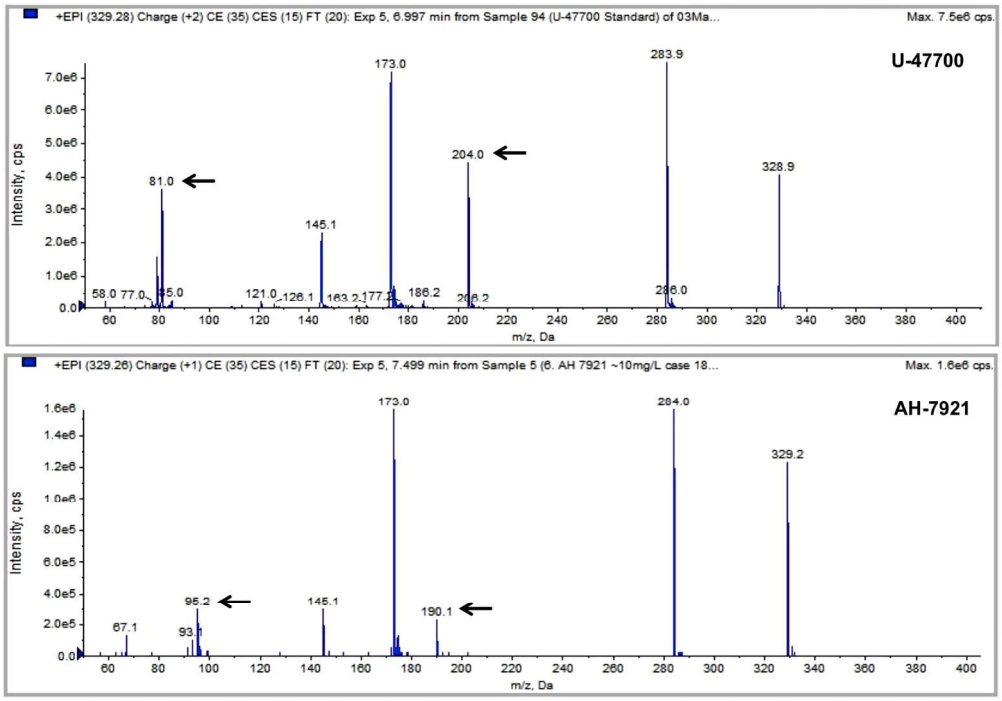

Enhanced product ion scans of U-47700 and AH-7921 using electrospray ionization HPLC linear ion trap mass spectrometry. Both structural isomers were differentiated based on distinct product ions as indicated by the arrows.
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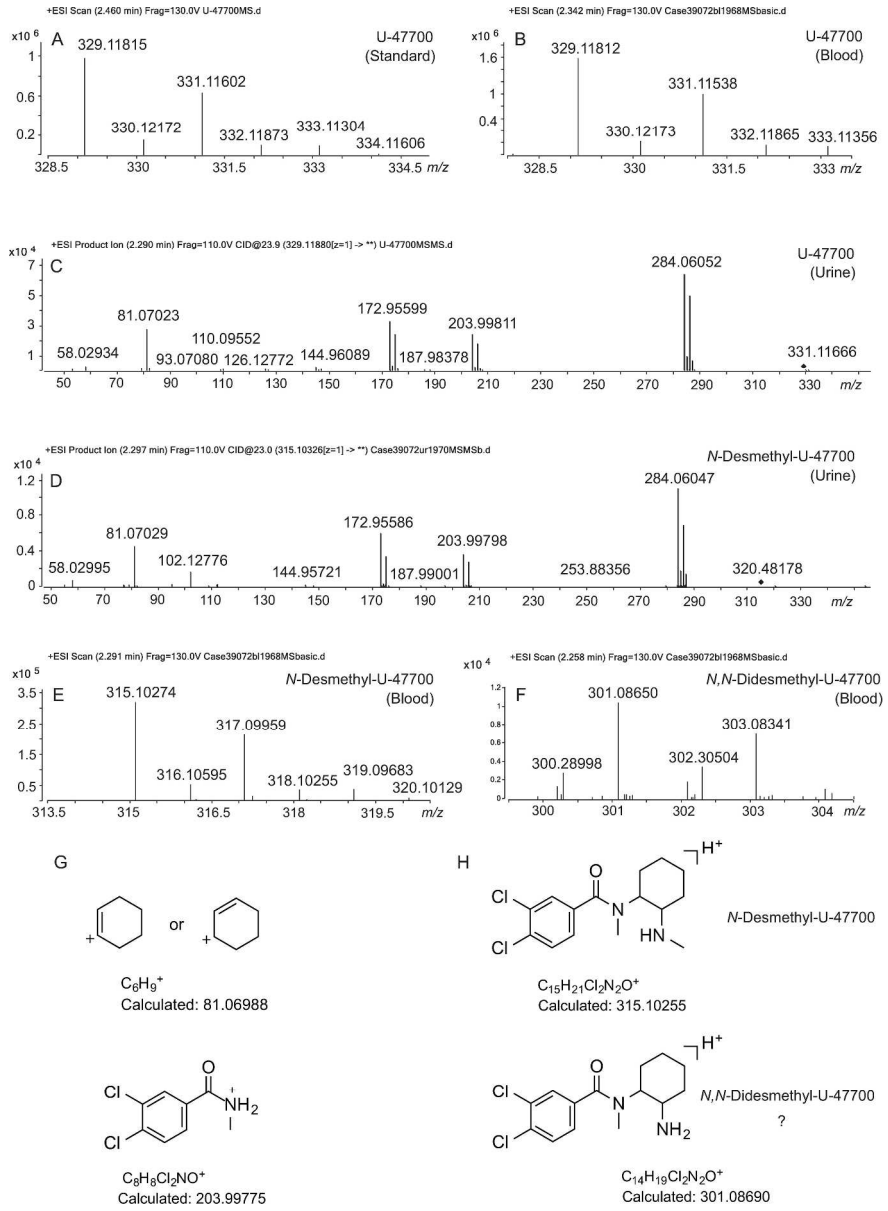

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284x390mm (300 x 300 DPI)