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Seven fatalities associated with ethylphenidate

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Abstract

Ethylphenidate is a stimulant novel psychoactive substance that is an analogue of the prescription drug methylphenidate (Ritalin®). Methylphenidate is used commonly for the treatment of attention deficit hyperactivity disorder. Due to its stimulant effects ethylphenidate is being abused. There is a single case report of a death associated with ethylphenidate in Germany, and a case series of 19 deaths in the East of Scotland, but otherwise, the contribution of ethylphenidate to death is poorly documented. We report the analytical results of 7 cases (between February 2013 and January 2015) in which ethylphenidate was detected and quantitated with a validated liquid chromatography tandem mass spectrometry method (LC-MS/MS). The individuals (all male) ranged in age from 23 to 49 years (median 25 years). The concentration of ethylphenidate in the cases ranged from 0.026 mg/L to 2.18 mg/L in unpreserved post-mortem femoral blood. Only one case had ethylphenidate present as a sole drug. All other cases had at least 2 other drug classes present (benzodiazepines, heroin, methadone antipsychotics, other new psychoactive compounds). Ethylphenidate toxicity was the sole contribution to the cause of death in one case. Hanging was the cause of death in 2 cases, with the other 4 cases being reported as having occurred due to mixed drug toxicity. These data will further help with the interpretation of post-mortem ethylphenidate levels.

Keywords: Ethylphenidate, Case Study, Methylphenidate, Poisoning, Post-mortem

1. Introduction

Ethylphenidate [ethyl 2-phenyl-2-(piperidin-2-yl) acetate; ritalinic acid ethyl ester; CAS: 57413-43-1 (base), 19716-79-1 (Hydrochloride) [Fig.1] is a racemic (+/- isomers) stimulant drug that is an analogue of the prescription drug methylphenidate (commonly known as Ritalin®). Ethylphenidate was first mentioned in the literature in 1961 [1] and was patented in 2003 [2] for the potential treatment of ADHD with reduced abuse potential when compared to methylphenidate. The abuse of methylphenidate having been recorded from the 1960's [3]. The first evidence of ethylphenidate abuse was in 2011 when it was officially reported to the European Monitoring Centre for Drugs and Drug Addiction (EMCDDA) after being found on sale in UK "headshops" [4]. Due to the reported abuse of ethylphenidate it was controlled in the UK under a temporary class order in April 2015 [5].

To date ethylphenidate has been sold in head shops and online under various names including "Nopaine", "Gogaine", "Ching" and "Burst (duck)" [6, 7], in various forms (powder, crystals and "pellets" (25 mg or 50 mg)) [7, 8], with a "recommended" oral dose of between 25 - 50 mg [8]. For insufflation users have reportedly used between 10 and 100 mg [9]. The effects of ethylphenidate that have been reported by users (stimulation, euphoria, an increase in focus, improved concentration, increased sociableness, empathy [8, 9]) are similar to those observed with other stimulants [10]. These "positive" effects are counteracted with the side-effects (anxiety; paranoia; hallucinations; increased heart rate blood pressure and body temperature; peripheral vasoconstriction; insomnia; sweating and nasal pain on insufflation [7-9, 11]) that are again commonly seen with other abuse stimulants [10].

The pharmacokinetics of ethylphenidate are unclear but information from user reports suggest that the mean onset time for ethylphenidate is ~ 13 min (range 0 – 35 min; n = 32) for nasal insufflation, and ~ 23 min (range 5 – 31 min; n = 8) for oral administration [8]. The mean duration of the effects were 117 min for all routes of administration (range 15 – 300 min; n = 29) however this is complicated by redosing. The half-life ($t_{1/2}$) of (-)-ethylphenidate can be estimated from the methylphenidate and ethanol human studies and is between 0.5 – 4.7h (mean ~1.25 h) n = 8 [12] although this may be complicated by the concomitant presence of methylphenidate and ethanol both of which are known to inhibit the metabolism of ethylphenidate and there may be also differences in metabolism of the (-) and (+) isomers of ethylphenidate [12-14]. Due to lack of clinical studies case reports are important to understand the toxicity of new psychoactive substances (NPS) such as ethylphenidate.

In this paper we report the toxicological findings of 7 cases where ethylphenidate was detected and quantitated and also for the first time report a death that was solely attributable to ethylphenidate. Detection and quantitation of ethylphenidate were carried out using liquid chromatography with tandem mass spectrometry (LC-MS/MS). These cases add to the body of knowledge of ethylphenidate and will help in the interpretation of cases where ethylphenidate is detected and quantitated.

2. Methods

2.1 Initial Toxicological Screening

All submitted toxicological samples underwent systematic toxicological analysis incorporating analysis for volatiles using headspace gas chromatography with flame ionisation detection (HS-GC-FID) and analysis for drugs by both gas chromatography-mass spectrometry (GC-MS) and liquid chromatography with tandem mass spectrometry (LC-MS/MS). The drugs were matched to both in-house (constructed from reference standards) and commercial libraries (NIST05 and SWGDRUG). Following confirmation of the presence of a drug the femoral blood drug levels were quantitated using LC-MS/MS.

2.2 Quantitation of ethylphenidate

2.2.1 Reagents and materials

Certified standards for ethylphenidate and d3-methylphenidate were purchased from LGC Standards (Teddington, UK). Methanol, chloroform, isopropanol, hydrochloric acid, 35% ammonia solution and glacial acetic acid were purchased from Fisher Scientific (Loughborough, UK). Potassium dihydrogen orthophosphate was purchased from BDH (Poole, UK). Strata Screen-C solid phase extraction (SPE) cartridges were purchased from Phenomenex (Macclesfield, UK). Blank equine serum was purchased from TCS Biosciences (Buckingham, UK).

2.2.2 Standards, calibrators, control and internal standard preparation

A stock solution of ethylphenidate was prepared at a concentration of 1 mg/mL in methanol and stored at -20 °C. A calibration curve between 0.005 – 1.00 mg/L was prepared in blank equine serum. The internal standard (d3-methylphenidate) was prepared at a concentration of 2 mg/L in methanol and stored at -20 °C. Calibrator materials were prepared prior to each extraction. If required, post-mortem blood samples were diluted in blank equine serum to fit into the calibration range. In-house quality control material was prepared from a separate stock solution of ethylphenidate at levels of 0.150 mg/L and 0.750 mg/L.

2.2.3 Extraction method for biological specimens

The extraction method was based on a standard solid phase extraction procedure [15]. Briefly, 1 mL of the standard/control/biological sample was added to a 10 mL glass tube and spiked with 200 µL of internal standard (d3-methylphenidate). 1 mL of phosphate buffer (0.1 M potassium dihydrogen orthophosphate in deionised water, pH 6.0) followed by 2 mL of deionised water was added to all tubes. Tubes were briefly vortexed and then sonicated for 10 minutes. After centrifugation at 3500 rpm for 8 minutes, the supernatant was added to Phenomenex Strata Screen-C solid phase extraction columns (pre-conditioned with methanol, deionised water and 0.1 M phosphate buffer) and eluted at low speed (1 - 2 mL/min). After washing with deionised water, 0.1 M hydrochloric acid and methanol, samples were eluted at low speed (1 - 2 mL/min) with 3 mL of elution buffer (800 mL chloroform, 200 mL isopropanol, 3 mL 35% ammonia solution) into 10 mL glass tubes. Samples were concentrated under nitrogen and reconstituted with 100µl of 10% methanol and transferred to a liquid chromatography vial for injection.

2.2.4 Instrumentation and LC-MS/MS parameters

Quantitative analysis of ethylphenidate was performed with a novel method using an Agilent Technologies 1200 Series HPLC system coupled with an Agilent Technologies 6460 Triple Quadrupole mass spectrometer. Agilent MassHunter Workstation Software B.03.01 was used for data analysis. Separation was carried out on an Agilent Zorbax SB-C18 30 mm × 2.1 mm column (particle size: 3.5 μm). A gradient elution program was applied at a flow rate of 0.6 mL/min with eluent A consisting of 0.1 % glacial acetic acid in deionised water and eluent B consisting of 0.1 % glacial acetic acid in methanol. Gradient elution was 0 – 1.5 min 10 → 100% B; 1.5 – 2.8 min 100% B; 2.8 – 4 min 100 → 10% B total run time of 4 minutes. Column temperature = 30 °C, injection volume = 2 μL. The mass spectrometer was operated in multiple reaction monitoring (MRM) mode using positive electrospray ionisation (ESI). Drying gas (nitrogen) temperature was set to 350 °C, gas flow rate to 5 L/min, nebuliser pressure to 45 psi, capillary voltage to 3500 V, collision energy (CE) to 20 V; dwell time to 30 ms; Vfr was set to 110 V for ethylphenidate and 100 V for the internal standard (d9-methylphenidate). The following ion transitions and retention times were observed: ethylphenidate (m/z 248/84 & 248/56), 0.976 min and the internal standard d9-methylphenidate (m/z 243/93 & 243/61), 0.720 min (quantitation ions underlined).

2.2.5 Method validation and matrix effects

The LC-MS/MS method for ethylphenidate quantitation was validated in plasma using the conditions previously described and according to the guidelines of Peters *et al.* [16]. Standard calibration curves for ethylphenidate in equine serum were linear from 0.005 – 1.00 mg/L with an $r^2 > 0.999$. Using quality control samples of 0.150 mg/L and 0.750 mg/L the method was shown to be both accurate and precise (interday (n=5) and intraday (n=30)) within acceptable ranges of ± 15%. The limit of detection (LOD) was found to be 0.002 mg/L and the lower limit of quantitation (LOQ) was found to be 0.005 mg/L. The matrix effects were evaluated by the methods of Matuszewski *et al.* [17] at both 0.150 mg/L and 0.750 mg/L and although ion suppression/enhancement was observed the results were within the accepted ± 15%.

3. Results and Discussion

This study reports the quantitative results of 7 post-mortem cases (between February 2013 and January 2015) across England in which ethylphenidate was detected and quantitated (table 1) using a validated LC-MS/MS method. These cases include a death where ethylphenidate was the sole drug detected, 2 cases in which drugs were not related to the cause of death (in this case hanging), and 4 cases where multiple drug toxicity was registered as the cause of death. The age range of the 7 individuals (all male) was 23 to 49 years (median 25 years), which is similar to the age ranges (20 – 54 years [6]; 32 & 38 years [18]) that was seen in previous cases reporting ethylphenidate deaths.

Table 2 lists the three previous publications in the literature that have given both analytically confirmed clinical and post-mortem concentrations of ethylphenidate. This gives some idea of the concentrations that can be observed in various case types. In the sole clinical case the measured blood concentration of ethylphenidate

was 0.24 mg/L, 20 hours after a reported use of 500mg of ethylphenidate [7]. In post-mortem samples that were not drug related (such as hanging and electrocution) the concentrations of ethylphenidate were 0.010 mg/L to >2.0 mg/L. In the two highest concentrations (1.3 mg/L and >2.0 mg/L) the causes of death were hanging (this study) and “a complication of the deceased’s intravenous drug administration, namely acute haemorrhage related to left groin abscess” [6]. They were not significantly lower than the sole case of ethylphenidate toxicity (2.2 mg/L) with no other drugs from this study (table 1). This with the other results presented in table 2 suggest that there may be a significant overlap between “therapeutic” and “fatal” levels of ethylphenidate.

The large variations of concentrations of ethylphenidate and their effects are likely to be both from natural variation in drug susceptibility and also tolerance to ethylphenidate, for example in one case where a user was reported to be taking up to fifty 20 mg pills per day [11]. Death due to ethylphenidate may also not be directly related to blood drug concentration and as with other stimulants ethylphenidate may cause cardiac damage [19]. In the first death cases related to ethylphenidate reported by Krueger *et al.*, it was suspected that the cardiovascular side effects may have contributed to the death especially as the deceased was found to have mitral valve endocarditis [18]. An added complication is that the concentrations of ethylphenidate may have been underreported due to instability, although ethylphenidate has not been subject to stability studies in blood or plasma, methylphenidate has been shown to be unstable in both blood and plasma due to hydrolysis by blood esterases [20-23]. From the results of these studies it appears that the best method of maintaining stability of ethylphenidate is most likely to be via storage at temperatures less than 4°C and in sodium fluoride (NaF) containing tubes [24] although this would need to be confirmed with stability studies. Finally it is a possibility post-mortem redistribution may have altered the drug concentrations in femoral blood after death [25]. The case results of ethylphenidate cases suggest that as with all toxicological cases the drug concentrations should be interpreted with the full knowledge of the case circumstances where possible. It is interesting to note that in both this study and the study by Parks *et al.*, a large number of individuals using ethylphenidate were also opiate abusers (heroin) 11 of 19 cases [6]; 3 of 7 cases [this study], overall >50% between the two studies. Parks *et al.*, also indicate that in 9 of the 17 reported cases ethylphenidate was being injected. Previous studies have utilised internet forum case reports to investigate the routes of administration of ethylphenidate [8, 9]. In the case reports ethylphenidate is most commonly insufflated (52.5%) followed by multiple routes (16.7%), oral ingestion (11.6%) and intravenous injection (9.6%). Minor routes include rectal insertion, sublingual insertion, smoking and intramuscular injection [8].

One of the major concerns with ethylphenidate that has been highlighted by the internet forum case studies is users reporting “a persistent impulse to redose” with 31 of 44 users redosing with ethylphenidate one or more times. The long term abuse potential is hard to determine without more detailed studies however the pharmacology of ethylphenidate suggests that there is a significant risk of abuse. Ethylphenidate exists as a racemic mixture with – (l) and + (d) isomers, pharmacological studies have shown that the individual isomers have different pharmacological activity [26, 27]. *In vitro* studies on human monoamine transporters 5-HT (SERT), noradrenaline (NET) and dopamine (DAT) expressed in HEK293 cells evaluated the pharmacology of the racemic mixture (-/+) and the individual isomers

have shown that, (+) ethylphenidate exhibited potent effects on DAT uptake inhibition ($K_i = 27$ nM), (-/+ ethylphenidate was slightly less potent ($K_i = 95$ nM) and (-) ethylphenidate was inactive (1730 nM). Both (+) and (-/+) ethylphenidate inhibited NET uptake although with less potency than DAT inhibition ($K_i = 230$ nM and 319 nM respectively) again (-) ethylphenidate was inactive [26]. None of the isomers had any functional activity on either dopamine 1-3 or 5-HT_{1A,2A,2C} receptors or on SERT uptake inhibition [26]. *In vivo* studies on mice showed that both (+) and (-/+) ethylphenidate cause significant increases in motor activity compared to control, when mice were administered 10mg/kg (i.p.) of ethylphenidate with (-) ethylphenidate showing again that it is inactive by having no significant effect on motor activity compared to control [26, 27]. The increase in locomotor activity is commonly used, among other *in vivo* tests (such as self-stimulation) as an indicator of abuse potential [28]. The increase in motor activity suggesting that ethylphenidate has abuse potential, further evidence can be found from the ratio of DAT/SERT inhibition with higher ratios thought to indicate a higher abuse potential [29]. From *in vitro* data [26] both (-/+) and (+) ethylphenidate have a DAT/SERT ratio of >105 indicating a high abuse potential, this is backed up by case studies where users of ethylphenidate feel the need to redose [8, 9].

Ethylphenidate, like its analogue methylphenidate has been shown to be initially metabolised to the pharmacologically inactive ritalinic acid (in phase I) by carboxylesterase 1C (hCES1c) [30], one of a group of carboxylesterases that commonly metabolise ester containing drugs (such as heroin and cocaine) [31]. Although full metabolic studies into ethylphenidate have not been carried out to date it is expected that ethylphenidate would follow a similar metabolic profile to methylphenidate [32]. The putative metabolic profile of ethylphenidate is shown in figure 1.

The first deaths that were related to ethylphenidate showed that ritalinic acid was detected in urine (both cases) along with methylphenidate (one case) and in blood (along with ethylphenidate) in both cases [18], the ritalinic acid in case 1 was 2.14 mg/L compared to 0.023 mg/L ethylphenidate. In case 2 ritalinic acid was 0.943 mg/L compared to 0.110 mg/L ethylphenidate. The high levels of ritalinic acid compared to ethylphenidate with no presence of methylphenidate or ethanol allow the confirmation of ethylphenidate as the drug that was initially taken and was not present as a metabolite. The fact that the metabolic interaction of ethanol and methylphenidate leads to the formation of ethylphenidate by transesterification was discovered following post-mortem analysis of two fatal overdoses [33]. Further controlled human studies were then carried out to investigate this phenomenon. The studies found that predominantly the inactive (-)ethylphenidate is formed, with the maximum amount of the (+)ethylphenidate only ever being 10% of the C_{max} concentration of (-)ethylphenidate, with (-)ethylphenidate only being around 10% of the concentration of methylphenidate plasma concentrations when concomitantly administered with ethanol [12-14]. The use of both enantiomer specific chromatography [34] and the levels of ethylphenidate detected with "normal" chromatography allow determination of the source of ethylphenidate. The studies [12-14] also showed that significant increases (~40% of mean) in the C_{max} concentration of methylphenidate were observed when methylphenidate is combined with ethanol, this adds confirmation that ethanol is an inhibitor of the hCES1 enzyme and as hCES1 is also responsible for the metabolism of ethylphenidate [30] that concomitant ethanol and ethylphenidate use would lead to higher blood

concentrations of ethylphenidate. In the course of the studies [12, 13] it was also determined that there are “poor” methylphenidate metabolisers in which ~100 times higher concentrations of methylphenidate were observed. Based on 29 subjects it is estimated that ~7% of people may be affected by being slow or poor metabolisers of methylphenidate (thought to be a CES1 null or reduced activity allele) and may exhibit increased concentrations of methylphenidate compared to “normal” metabolisers.

Polymorphisms have been found in hCES1 [35] but have yet to be evaluated in relation to metabolism of either methyl or ethylphenidate. It is likely as the same enzyme metabolises both ethyl and methylphenidate that ethylphenidate would also be affected [30], and that concomitant administration of a drug such as heroin (which is also metabolised by hCES1a [30]) and other potent inhibitors of carboxylesterase such as ethanol, digitonin, telmisartan [36], aripiprazole, fluoxetine [37] and loperamide [38] could also cause increases in the blood levels of ethylphenidate if taken concomitantly, potentially to fatal levels.

These results and the literature review show that more information is required about both the pharmacology and toxicology of ethylphenidate in order to understand the effects that it has on the human body.

Figure 1: Putative metabolism of ethylphenidate

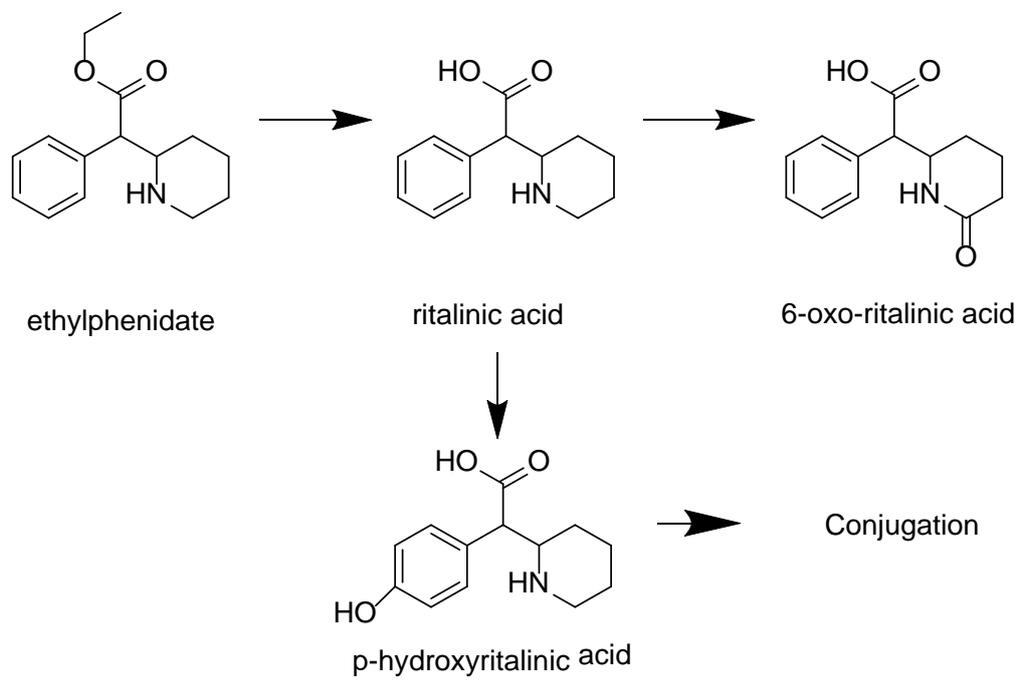


Table 1: Ethylphenidate concentrations and cause of death

Ethylphenidate concentration (mg/L)	Certified Cause of death	Other drugs detected
2.18	Ethylphenidate toxicity	None
1.37	Hanging	Benzoyllecgonine = 0.012 mg/L Sertraline = 0.295 mg/L Diphenhydramine = 0.043 mg/L
0.87	Hanging	Dothiepin = 0.017 mg/L Methiopropamine = 4.640 mg/L Ethanol = 74 mg/100mL
0.11	Methadone toxicity + 2 amino indane toxicity	Methadone = 0.807 mg/L EDDP = 0.532 mg/L Zopiclone = 0.123 mg/L Sertraline = 0.494 mg/L Aripiprazole = 0.073 mg/L Dehydroaripiprazole = 0.016 mg/L 2-Aminoindane = 0.101 mg/L Ethanol = 30 mg/100ml
0.14	Heroin toxicity	Morphine (free) = 0.117 mg/L Morphine (total) = 0.180 mg/L Codeine (free) = 0.011 mg/L Ketamine = 0.518 mg/L Cocaine = 0.12 mg/L Benzoyllecgonine = 0.272 mg/L Venlafaxine = 0.344 mg/L O-desmethylvenlafaxine = 0.374 mg/L
0.03	Mixed drug toxicity	Methiopropamine = 0.051 mg/L 5APB/6APB = 1.410 mg/L
0.11	Multiple drug toxicity	Diazepam = 0.316 mg/L Nordiazepam = 0.409 mg/L Temazepam = 0.017 mg/L Oxazepam = 0.009 mg/L Morphine (free) = 0.071 mg/L Morphine (total) = 0.101 mg/L Codeine (free) = 0.014 mg/L

Table 2: Ethylphenidate concentrations found in a variety of case types (clinical, drugs deaths, not drug related) in blood/serum/plasma

Author	Case Type	Number of Cases	Ethylphenidate Concentration (mg/L)
Krueger <i>et al.</i> , [18]	Not drug related	2	0.023, 0.110 (femoral blood)
Park <i>et al.</i> , [6]	Not drug related	6	0.010 - >2.0 (femoral blood)
This Study	Not drug related	2	0.87, 1.37 (femoral blood)
Bailey <i>et al.</i> , [7]	Clinical overdose	1	0.24 (blood)
Park <i>et al.</i> , [6]	Antemortem	2	0.030, 0.46 (antemortem)
This Study	Drug toxicity (not related to ethylphenidate)	2	0.11, 0.14 (femoral blood)
Park <i>et al.</i> , [6]	Drug toxicity (not related to ethylphenidate)		0.010, 0.040 (femoral blood)
Park <i>et al.</i> , [6]	Drug toxicity (multiple)	6	0.25 - 1.9, (femoral blood)
This Study	Drug toxicity (multiple)	2	0.03, 0.11 (femoral blood)
This Study	Ethylphenidate Toxicity	1	2.2 (femoral blood)

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