Studies on the Metabolism and Toxicological Analysis of the new Designer Drug α-Pyrrolidino-valerophenone (PVP) in Rat Urine Using GC-MS Techniques

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Abstract

The aim of the presented study was to identify the metabolites of the new designer drug α-Pyrrolidinovalerophenone (PVP) in rat urine using GC-MS techniques and to identify the CYP isoenzymes involved in the main metabolic step. Eleven metabolites of PVP could be identified suggesting the following metabolic steps: hydroxylation of the side chain followed by dehydrogenation to the corresponding ketone; hydroxylation of the 2''-position of the pyrrolidine ring followed by dehydrogenation to the corresponding lactam; degradation of the pyrrolidine ring to the corresponding primary amine; hydroxylation of the phenyl ring, most probably in the 4'-position; ring opening of the pyrrolidine ring to the corresponding carboxylic acid. The authors´ screening procedure for pyrrolidinophenones allowed detection of PVP metabolites. Additionally, investigation of the CYP dependent side chain hydroxylation revealed the involvement of the following CYP isoenzymes in this main metabolic step: 2B6, 2C19, 2D6, and 3A4.

1. Introduction

1-Phenyl-2-pyrrolidin-1-ylpentan-1-one (α-pyrrolidinovalerophenone, PVP) is a new designer drug of the pyrrolidinophenone type. It was the latest of a series of structurally related compounds of this class of designer drugs to occur on the illicit drug market in Germany. The pyrrolidinophenones are distributed among drug abusers as tablets, capsules, or powders [2].

Little information is available about the dosage and the pharmacological and toxicological effects of the pyrrolidinophenones. However, they may be expected to be very similar to those of pyrovalerone (4´-methyl-α-pyrrolidinovalerophenone) acting by releasing dopamine and norepinephrine from the respective nerve terminals [6, 15]. In 2006, Meltzer et al. synthesized series of pyrovalerone analogues, among them PVP, which were tested concerning their biological activity at dopamine, serotonin and norepinephrine transporters. Pyrovalerone was withdrawn from the market and scheduled as a controlled substance after reports of its intravenous abuse by polytoxicomaniacs [4]. While the metabolism of other pyrrolidinophenones has been described, PVP has never been studied in this respect.

The involvement of particular CYP enzymes in the biotransformation of a new chemical entity is usually thoroughly investigated before it can be marketed. Such investigations allow the prediction of possible drug-drug-interactions, in the case of co-administration of a drug that is a substrate itself or influences the corresponding enzyme (i.e. induces or inhibits). Furthermore, some CYPs (e.g. CYP2D6) are expressed polymorphically in humans and metabolism by these enzymes can therefore lead to inter-individual variations in pharmacokinetic profiles and an increased appearance of side effects and serious poisonings [5]. However, such risk assessment is typically performed for substances intended for therapeutic use, but not for drugs of the illicit market. So far no data are available on the CYP mediated metabolism of PVP.

Therefore, the aim of the presented study was to identify the PVP metabolites in rat urine using GC-MS in the electron impact (EI) and positive-ion chemical ionization (PICI) mode, to use this knowledge for incorporation of PVP into authors´ EI GC-MS-based toxicological screening procedure for the other pyrrolidinophenone-type designer drugs in urine [20, 23] and to investigate the involvement of human CYP isoenzymes in the side chain hydroxylation of PVP.

2. Experimental

2.1 Chemicals and reagents

PVP-HNO_3 from a drug seizure was provided by Hessisches Landeskriminalamt, Wiesbaden (Germany) for research purposes. Bond Elut cartridges (130 mg, 3 mL) were obtained from Varian (Darmstadt, Germany). *N*-Methyl-*N*-trimethylsilyl-trifluoroacetamide (MSTFA), trifluoroacetic anhydride (TFA), and heptafluorobutyric anhydride (HFBA) were obtained from Fluka (Steinheim, Germany). Diazomethane was synthesized according to the procedure of McKay et al. [10]

2.2 Urine samples

The investigations were performed using urine of male rats (Wistar, Charles River, Sulzfleck, Germany) for toxicological diagnostic reasons according to the corresponding German law. They were administered a single 20 mg/kg body mass dose of PVP- HNO_3 for identification of the metabolites and a 1 mg/kg body mass dose of PVP-HNO_3 for toxicological analysis. For details see reference [14].

2.3 Sample preparation

The samples were worked up as described in reference [14]. Briefly, a 0.5 mL portion of urine was incubated with 50 µL of a mixture of glucuronidase and arylsulfatase from *Helix Pomatia L*. The urine sample was then diluted with 2.5 mL of water and loaded on a Bond Elut Certify cartridge previously conditioned with 1 mL of methanol and 1 mL of water. After passage of the sample, the cartridge was washed with 1 mL of water, 1 mL of 0.01 M hydrochloric acid, and again with 1 mL of water. The retained non-basic compounds were first eluted into a 1.5 mL reaction vial with 1 mL of methanol (fraction 1), whereas the basic compounds were eluted in a second step into a different vial with 1 mL of a freshly prepared mixture of methanol/aqueous ammonia 32% (98:2 v/v, fraction 2). The eluates were gently evaporated to dryness under a stream of nitrogen at 56°C and derivatized by methylation, acetylation, combined methylation/acetylation, or trimethylsilylation according to published procedures [7]. The same derivatizations were repeated without the use of enzymatic hydrolysis to study which metabolites of PVP were excreted as glucuronides/sulfates.

2.4 GC/MS analysis

The extracts were analyzed using a Hewlett Packard (Agilent, Waldbronn, Germany) 5890 Series II gas chromatograph combined with an HP 5989B MS Engine mass spectrometer and an HP MS ChemStation (DOS series) with HP G1034C software version C03.00. The GC conditions were as follows: splitless injection mode; column, HP-1 capillary (12 m x 0.2 mm I.D.), cross linked methyl silicone, 330 nm film thickness) and the MS was operated in EI and PICI full scan mode. For details see references [12, 14].

2.5 Microsomal Incubation

The microsomal incubations were performed as described in reference [21]. Briefly, mixtures (final volume: 50 µL) consisted of 90 mM phosphate buffer (pH 7.4), 5 mM Mg^{2+}, 5 mM isocitrate, 1.2 mM NADP+, 0.5 U/mL isocitrate dehydrogenase, 200 U/mL superoxide dismutase and substrate at 37°C. Reactions were started by addition of ice-cold microsomes and terminated with 5 µL of 60% (w/w) perchloric acid. Incubations were performed with 50 µM PVP and 50 pmol/mL CYP1A2, CYP2A6, CYP2B6, CYP2C8, CYP2C9, CYP2C19, CYP2D6, CYP2E1, or CYP3A4 for 30 min.

PVP and HO-PVP were separated and determined using an Agilent Technologies (AT, Waldbronn, Germany) AT 1100 series atmospheric pressure chemical ionisation (APCI) electrospray LC-MSD, SL version and an LC-MSD ChemStation using the A.08.03 software. Gradient elution was achieved on a Merck LiChroCART® column (125 x 2 mm I.D.) with Superspher®60 RP Select B as stationary phase and a LiChroCART®10-2 Superspher®60 RP Select B guard column. The mobile phase consisted of ammonium formate (50 mM, ad-

justed to pH 3.5 with formic acid) (eluent A) and acetonitrile containing 1 mL/L formic acid (eluent B) according to reference [8]. The gradient and the flow rate were programmed as follows: The gradient and the flow rate were programmed as follows: 0-4 min 40% B (flow: 0.4 mL/min). The injection volume was 5 µL. The following APCI inlet conditions were applied: drying gas, nitrogen (7000 mL/min, 300°C); nebulizer gas, nitrogen (172.3 kPa); capillary voltage, 4000 V; vaporizer temperature set at 400°C; corona current was 5.0 µA; positive selected-ion monitoring (SIM) mode; fragmentor voltage 50 V.

3. Results and Discussion

3.1 Identification of Metabolites

The urinary metabolites of PVP were identified by full-scan EI and PICI MS after GC separation. The postulated structures of the metabolites were deduced from the fragments detected in the EI mode which were interpreted in correlation to those of the parent compound according to the rules described by e.g. McLafferty and Turecek [11] and Smith and Busch [17]. In order to verify the molecular mass of the postulated metabolites, PICI mass spectra were recorded because they contain strong molecular peaks ($M+H^+$), in contrast to EI spectra

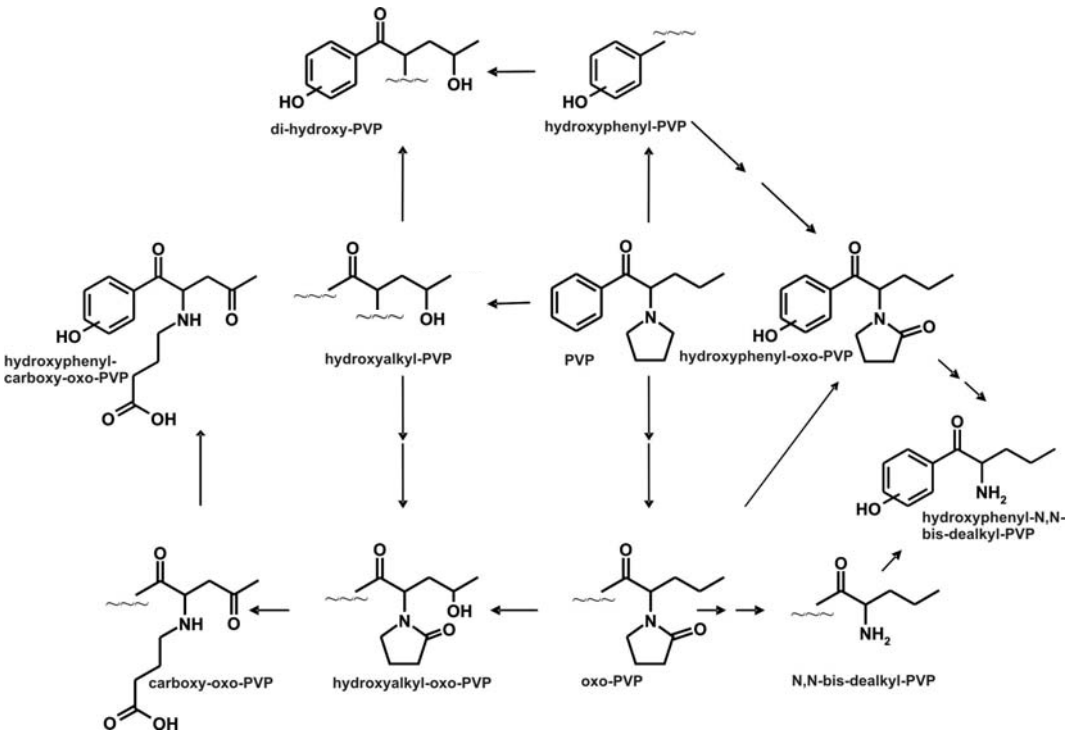

Figure 1 Proposed scheme for the metabolism of PVP in rats.

EI and PICI mass spectra, the gas chromatographic retention indices (RI) determined according to ref. [3], structures and predominant fragmentation patterns of PVP as well as of its acetylated, methylated, trifluoroacetylated, and trimethylsilylated metabolites can be found in references [9, 14]. From these mass spectra, metabolites could be deduced, whose structures are given in the postulated metabolic pathway shown in Fig.1. For details see reference [14]. As the peaks of HO-phenyl-PVP, hydroxyphenyl-oxo-PVP, di-HO-PVP, HO-phenyl-amino-PVP, and HO-alkyl-PVP were more abundant after glucuronidase and sulfatase hydrolysis, it can be concluded that they were partly excreted as glucuronides and/or sulfates

Concerning the structures of the metabolites, it must be mentioned that the exact position of the oxo group in the pyrrolidino-oxo metabolites of PVP could not be deduced from the fragmentation patterns. In previous studies on the metabolism of other pyrrolidinophenones [13, 16, 18-20, 22, 23], introduction of an oxygen atom in position 2'' leading to the respective lactams had been considered most likely. This assumption was based on the fact that lactam formation is common in the metabolism of other pyrrolidino compounds such as prolintane and nicotine [24]. The present study is the first in which carboxy metabolites corresponding to the hydrolyzed lactam ring were detected. Since such metabolites can only be considered either as precursors or hydrolysis products of respective lactams, their existence provides very strong evidence that the oxo group of the oxo metabolites is indeed in position 2''. Identification of the exact position of the hydroxy group in the side chain could also not be deduced from fragmentation patterns. A similar problem had previously been described for the side-chain homologue MPHP [23]. However, in a recent paper [1], it was shown that short alkyl chains are preferentially hydroxylated in position ω-1, i.e. at the penultimate carbon atom. Assuming that this was also the case for PVP side chain hydroxylation, the oxo groups resulting from the side chain hydroxy metabolites would also be in position ω-1. In this position, they would be able to form a six-membered ring forming a hydrogen bond with the nitrogen atom in the carboxy-oxo metabolites. Such rings being rather stable, this could possibly explain why such carboxy metabolites were only observed in the case of PVP. In the related pyrrolidinophenones MPBP and MPHP, a carbonyl group in position ω-1 would either be to close or to far away from the nitrogen to form such a stable nitrogen bond. In our opinion, these findings clearly indicate hydroxylation of the PVP side chain indeed occurs in position ω-1, although they are no definitive proof of this assumption.

3.2 Toxicological Detection by GC-MS

PVP and its metabolites were separated by GC and identified by full-scan EI MS after fast enzymatic hydrolysis, SPE and trimethylsilylation according to reference [18-20, 22, 23]. In the trimethylsilylated extract of rat urine after administration of 1 mg/kg body mass dose of PVP-HNO_3, the main metabolites

detected are the oxo- and the hydroxyphenyl-*N*,*N*-bis-dealkyl metabolite which allows the detection of an intake of PVP in the urine of drug abusers, because the above mentioned 1 mg/kg body mass dose of PVP should approximately correspond to a dose ingested by abusers. Mass chromatography with the following ions was used to detect the presence of these metabolites: *m/z* 126, 140 and 144. The selected ion *m/z* 126 was used for monitoring the presence of compounds with unchanged pyrrolidine ring, *m/z* 140 for compounds with oxidized pyrrolidine ring and *m/z* 144 for the amino-metabolites. The identity of the peaks in the mass chromatogram was confirmed by computerized comparison of the underlying mass spectrum with reference spectra recorded during this study [17]. For details see reference [14].

3.3 Microsomal Incubation

The initial screening studies with the nine most abundant human hepatic CYPs were performed to identify their possible role in PVP side chain hydroxylation. According to the supplier's advice, the incubation conditions chosen were adequate to make a statement on a general involvement of a particular CYP enzyme. The data revealed that CYP2B6, CYP2C19, CYP2D6 and CYP1A2 were markedly capable of the side chain hydroxylation of PVP (Fig. 2). In addition to that, CYP2C19 and CYP2D6 are two CYPs which are expressed polymorphically in humans.

4. Conclusion

The presented studies showed that the new designer drug PVP was extensively metabolized by rats via several pathways. Therefore, the urine screening procedure should be focused on the metabolites. Assuming similar metabolism and dosages in humans, an intake of PVP should be detectable via its metabolites in urine. Additionally, CYP2B6, CYP2C19, CYP2D6 and CYP3A4 could be identified as the CYP isoenzymes involved in the main metabolic step of PVP metabolism.

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6. References

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