Target Urinary Analytes for the Gas Chromatographic–Mass Spectrometric Detection of Procyclidine and Benzhexol in Drug Abuse Cases

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Abstract

The two antiparkinsonian drugs procyclidine and benzhexol are presently finding considerable favor for their euphoric hallucinogenic effects among drug abusers in some countries. In anticipation of their possible scheduling in national drug laws, gas chromatography–mass spectrometry (GC–MS) methods for their detection in urine will be required. However, because of uncertainty of the metabolic fate of the two drugs in humans, the urinary target analytes for GC–MS detection were not well defined. The problem was addressed in the present study in which it was found that mono-hydroxy metabolites, where hydroxylation took place at the cyclohexane ring in both drugs, could be endorsed as the major target analytes. The metabolites could only be detected as the mono- and/or di-trimethylsilyl (TMS) derivatives. The predominance of either derivative depended on the temperature and time of heating with the derivatizing reagent. Because of the basic properties of the hydroxy metabolites, analytic method optimization was needed for their detection in urine included extraction under basic pH conditions. Urine hydrolysis with β-glucuronidase did not have an effect on the recovery of the metabolites, but was usually performed in search for other drugs. Because of the relative abundance of ions, the electron impact mass spectra of the mono-TMS derivatives and the chemical ionization (CI) mass spectra of the mono- and di-TMS derivatives of the hydroxy metabolites of both drugs were found to be more structurally informative. The CI mass spectra of the di-TMS derivatives have the additive advantage of being potentially useful for quantitative analysis.

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Introduction

Procyclidine and benzhexol (Figures 1 and 2) are structurally related tertiary amine antimuscarinic drugs used in the treatment of Parkinsonism. They are also employed in heroin dependence treatment programs to control tremors associated with heroin withdrawal. Not surprisingly, their use in the latter respect has led to their abuse, because they may produce euphoric hallucinogenic effects upon using higher than therapeutic doses (1–7). In fact, their use in combination with tramadol, benzodiazepines, and carisoprodol is currently finding great favor among drug abusers in this country, mainly because of the shortage in heroin supply, easy access from smuggled sources, and diversion of legal prescription. The extent of such abuse during the past four years is shown in Table I. Accordingly, and in anticipation of the scheduling of these drugs in the national drug laws of the UAE and possibly other countries, there was need for recognizing target analytes in urine and optimizing methods for their detection. Indeed, gas chromatography–mass spectrometry (GC–MS) came to the fore for satisfying such needs. In fact, Hadidi (8) reported a GC–MS method for the screening of benzhexol and procyclidine in plasma and urine based on the unchanged forms of the drugs. However, in the absence of knowledge about the major forms in which a drug is found in the urine, development of analytical methods based on the unchanged form of the drug may not be the best approach as sensitivity may be compromised.

The information available in the literature about the disposition of procyclidine and benzhexol in humans threw little light on the major forms in which the drugs were detected in urine. For both drugs, hydroxylation was reported to occur at different sites in the aromatic or the cyclohexane ring. For procyclidine, Ashton (9) suggested metabolic *p*-hydroxylation

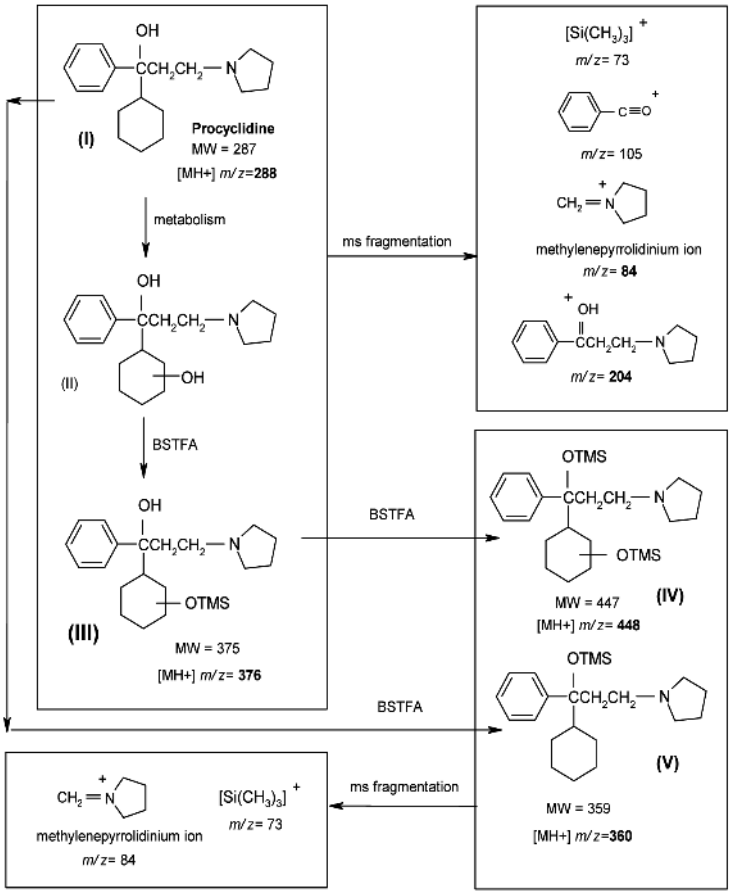

Figure 1. Procyclidine metabolism, TMS derivatization, and proposed major MS fragmentation ions in the electron impact mode.

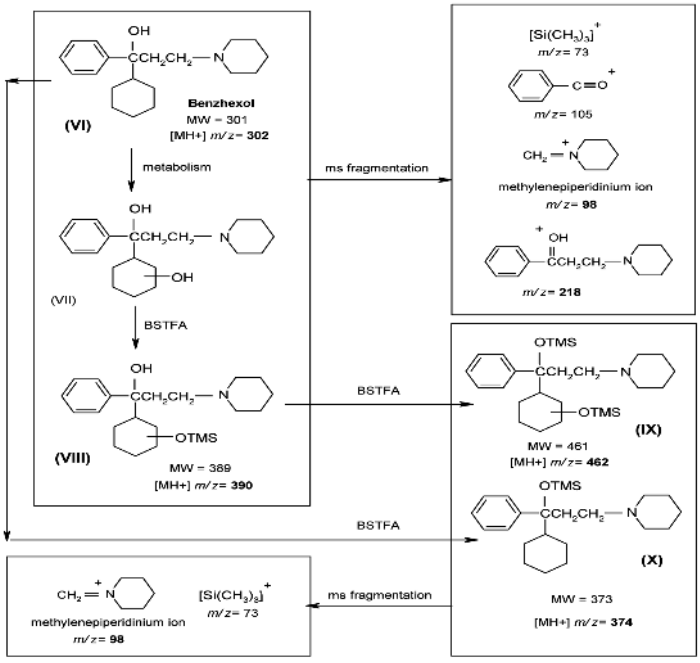

Figure 2. Benzhexol metabolism, TMS derivatization, and proposed major MS fragmentation ions in the electron impact mode.

of the benzene ring, although Paeme et al. (10) suggested meta-hydroxylation of the cyclohexane ring. Dawling and Robins (11) and Kintz et al. (12) reported significant amounts of both the parent drug and a hydroxylated metabolite in urine. Nation et al. (13) reported a series of hydroxylated metabolites for benzhexol. Generally, the occurrence of metabolic hydroxylation and its site in a xenobiotic molecule will have an impact on the development of a GC–MS analytical method for the resulting metabolite regarding sample hydrolysis, extraction, and derivatization. Accordingly, the objective of the present work was to identify the urinary target analytes of procyclidine and benzhexol and optimize the GC–MS conditions for their detection.

Materials and Methods

Materials

Benzhexol and procyclidine were obtained by preparative TLC from powdered Artane® and Kemadrin® tablets, respectively. The identity and purity of each compound was tested by GC–MS and UV. The tablets were obtained from the Pharmacy of Sharjah Police Private Rehabilitation Centre (Sharjah, UAE).

Urine samples of users of the two drugs were obtained from Sharjah Police Rehabilitation Centre where the two drugs are used in drug treatment programs for heroin addiction. Bis(trimethylsilyl)trifluoroacetamide/1% trimethylchlorosilane (BSTFA/1% TMCS) and β-glucuronidase (type HP-2, from *Helix pomatia*) were purchased from Sigma-Aldrich (Deisenhofen, Germany). Toxi-Tubes A for basic drug extraction were obtained from Varian (Walnut Creek, CA). Other reagents used were of analytical grade.

Procedures

GC–MS of reference procyclidine and benzhexol

A 2-µL aliquot of an approximately 2 µg/mL methanolic solution of either procyclidine RS or benzhexol RS was injected into the GC–MS. After evaporation of methanol from the remaining solution, the residue was heated with 100 µL of BSTFA/1%TMCS at 60°C for 10 min

and a 2-µL aliquot was injected into the GC–MS.

Urine sample preparation for GC–MS analysis

Without enzyme hydrolysis. Urine (3 mL), containing 500 µL of 1 M acetate buffer (pH 4.8) and 60 µL of water, was heated at 56°C for 60 min and, after cooling, was extracted with a Toxi-Tube-A by mechanical shaking for 10 min. The tube was then centrifuged at 3000 rpm (1687 × *g*) for 5 min, the organic layer was separated, and the solvent was evaporated under nitrogen at 40°C. The residue was dissolved in methanol (100 µL) and a 2-µL aliquot was injected into the GC–MS. After evaporation of the methanol from the remaining solution under nitrogen at 40°C, the residue was heated with BSTFA/1% TMCS (100 µL), and a 2-µL aliquot of the solution was injected into the GC–MS. The heating with the derivatizing reagent was performed at three temperatures, 40°C, 50°C, and 60°C, for three lengths of time, 5, 10, and 20 min, at each temperature.

Another residue, obtained in the same manner as described previously, was heated with of a 2:1 mixture of acetic anhydride/pyridine (500 µL) at 60°C for 30 min. After evaporation of the excess derivatizing reagent under nitrogen at 50°C, the residue was dissolved in methanol (100 µL), and a 2-µL aliquot was injected into the GC–MS.

With enzyme hydrolysis. Five-hundred microliters of 1 M acetate buffer (pH 4.8) and 60 µL (6642 units) β-glucuronidase were added to the urine sample (3 mL) in a screw-capped glass vial; the vial was heated at 56°C for 60 min. After cooling, the mixture was extracted with Toxi-Tube-A for basic drug extraction. The solvent was then evaporated off the organic layer under nitrogen at 40°C, the residue was heated with BSTFA/1% TMCS (100 µL), and a 2-µL aliquot was injected into the GC–MS. The heating with the derivatizing reagent was performed at three temperatures of 40, 50, and 60°C and three heating times of 5, 10, and 20 min at each temperature.

Another residue, obtained in the same manner as described previously, was heated with a 2:1 mixture of acetic anhydride/pyridine (500 µL) at 60°C for 30 min. After evaporation of the excess reagent, the residue was dissolved in methanol (100 µL), and a 2-µL aliquot was injected into the GC–MS.

GC–MS and analytical conditions

A Saturn 2200 GC–MS system from Varian was used in both electron impact (EI) and chemical ionization (CI) modes of analysis. The system consisted of a Varian 3800 GC interfaced with ion trap Saturn MS 2200, a capillary 1177 split/splitless injector and a Varian 8200 autosampler device. Analysis was performed on a 5% phenyl 95% dimethylpolysiloxane (30 m × 0.25 mm × 0.25-µm film thickness) column (CP-Sil 8 CB Low Bleed MS, Varian). A 2-µL splitless injection was performed with the following oven program: an initial temperature of 100°C held for 2 min, then ramped at 10°C/min to 190°C and at 20°C/min to 280°C with a final hold time of 14 min. The carrier gas was helium with a flow rate of 1 mL/min. The injector and interface temperatures were set at 250°C and 260°C, respectively. A scan range of 47–650 was used at a scan rate of 2 scans/s. The ion trap temperature was set at autotune value and the target TIC was 5000 counts.

For EI ionization, the filament current was set at 10 µA and the maximum ionization time was 15,000 µs with a background mass of *m/z* 45.

For CI, the filament current was set at 20 µA and the storage level was *m/z* 19. The background mass was *m/z* 65 with the maximum ionization time of 2000 µs and a maximum reaction time of 60 ms. The reagent gas was isobutane with a pressure of 5 psi.

GC–EI-MS analyses were also conducted on an Agilent (HP) series 6980 GC interfaced with a 5973 HP mass selective detector (Palo Alto, CA) using an HP-5MS capillary column (cross-linked 5% phenyl-methyl polysiloxane, 30 m × 0.25-mm i.d., 0.25-µm film thickness; Hewlett-Packard, Palo Alto, CA). The injector port and transfer line were maintained at 250°C and 280°C, respectively. Helium was used as carrier gas at a flow rate of 1 mL/min. Splitless injection of a 2-µL extract was performed with the following oven temperature program: the initial temperature was 100°C, held for 2 min, and then raised at 20°C/min to 280°C with a final hold time of 5 min. EI ionization analysis was performed with ionization energy at 70 eV. Data were acquired in the scan mode in a range of *m/z* 50–550 at a rate of 2 scans/s. The ion source temperature was 230°C.

Table I. Percentage of Combination Use of Procyclidine and Benzhexol with Benzodiazepine and/or Tramadol in a Four-Year Period (2005–2009)

Drug*	Benzhexol	Procyclidine	Benzodiazepine	Tramadol	% of 600 Urine Samples Screening
Benzhexol	√				0
		√			1
	√	√		√	0
			√		7
		√	√		3
				√	6
			√	√	23
		√	√	√	11
Procyclidine		√			0
			√		5
				√	4
			√	√	40

* Alone or in combination with benzhexol, procyclidine, benzodiazepine, or tramadol.

Results and Discussion

In heroin-dependence treatment programs, procyclidine and benzhexol are usually used in combination with the benzodiazepine group and the narcotic analgesic tramadol. Likewise, in cases of abuse, combination use of procyclidine

or benzhexol with benzodiazepines and tramadol is widespread. As far as could be determined, the benzodiazepines mostly prescribed and abused included, in order of increasing preference, bromazepam, diazepam, clonazepam, midazolam, and alprazolam. In the laboratory, the GC–MS confirmation of urine immunoassay positive results for benzodiazepines involved β-glucuronidase hydrolysis, solvent extraction, and TMS derivatization as essential presteps. Accordingly, the GC–MS detection of procyclidine and benzhexol under such conditions needed to be investigated.

The results in Table II, obtained on the Varian ion-trap system, represent GC–EIMS and GC–CIMS analysis of procyclidine and benzhexol as reference standards and in urine after TMS derivatization. The data were obtained by extracting ion-chromatograms at *m/z* 84 for procyclidine and 98 for benzhexol. The two values, which are derived from methylenepyrrolidinium and methylenepiperidinium ions (Figures 1 and 2), represent the base-peak ions in the mass spectra of compounds that contain the appropriate precursor chemical grouping, such as procyclidine and benzhexol. It was known that when such ions could be generated by mass fragmentation, they would dominate the mass spectra (14). It should be emphasized that, based on previous literature reports on the metabolism of procyclidine and benzhexol (9–13), the assumption was made that no metabolic changes had taken place at the ethylenepyrrolidine or ethylenepiperidine moieties from which the base-peak ions of procyclidine and benzhexol were derived, respectively.

Characterization of the target analytes of procyclidine and benzhexol in urine

The compounds discussed in this section are numbered from I to V for procyclidine and from VI to X for benzhexol. The GC–MS data of the compounds are given in Table II, and the corresponding chemical structures are given in Figures 1 and 2. Whenever reference is made to a compound by its number, reference should be made to Table II and Figure 1 or 2 as appropriate.

The two drugs reference standards were detected in the free forms (compounds I and VI) and the TMS-derivatized forms (compounds V and X) as indicated by the difference of 72 in molecular weights (359–287 for procyclidine and 373–301 for benzhexol), which represented TMS derivatization of the intrinsic hydroxy group.

Generally, phase I metabolic hydroxylation of a xenobiotic results in molecular weight increase by 16 mass units. TMS

Table II. Ion-Trap GC–MS Data for Procyclidine and Benzhexol Reference Standards and in Urine*,†,‡

Reference Standard			Urine		
RT (min) (Compound no.)	*m/z* EIMS	*m/z* CIMS	RT (min) (Compound no.)	*m/z* EIMS	*m/z* CIMS
Procyclidine					
20.53 (**I**)	84 (100), 204 (15), 205 (8.3) 105 (3.1) 77 (3.1)	**288** (100), 84 (57)	22.43 (**III**)	84 (100) 73 (12.4) 204 (9.3) 205 (7.0) 105 (6.2)	376 (100) 84 (23.9) 286 (14.0) 205 (4.0) 204 (4.0)
19.73 (**V**)	84 (100) 73 (7.3)	84 (100), **360** (18.3)	21.28 (**IV**)	84 (100) 73 (26) 432 (6.4)	84 (100) 432 (30.8) **448** (24.4) 358 (5.1) 259 (5.1)
Benzhexol					
21.13 (**VI**)	98 (100), 218 (9.3), 219 (3.5) 105 (3.1) 77 (3.1)	**302** (100), 98 (55.3), 218 (4.7)	22.98 (**VII**)	98 (100) 73 (12.5) 219 (19.3) 218 (16.3) 105 (5.2)	**390** (100) 98 (52.8) 300 (43.4) 219 (13.2)
20.35 (**X**)	98 (100) 73 (7.3)	98 (100), **374** (27.9)	21.82 (**VIII**)	98 (100) 73 (26.5)	98 (100), **462** (30.2) 446 (22.6) 318 (9.4) 259 (7.5) 372 (5.7)

* Analyses were done after TMS derivatization.
† Compounds II and IX (Figures 1 and 2, respectively) are the underivatized hydroxy metabolites of procyclidine and benzhexol, respectively, so they do not feature in the table because they are not detected with this analytical scheme.
‡ Values in bold are referenced in the Characterization of the target analytes subsection.

derivatization of the metabolically introduced hydroxyl group will add a further 72 mass units to the molecular weight, thus bringing the total increase to 88 mass units (16 + 72). Accordingly, from the difference of 88 in the molecular weights of compounds III and I (375–287) and compounds VIII and VI (389–301), compounds III and VIII were TMS-derivatized hydroxy metabolites of procyclidine and benzhexol, respectively. This was further substantiated by the same difference (i.e., 88) in molecular weights between compounds IV and V for hydroxyprocyclidine (447–359) and compounds IX and X for hydroxybenzhexol (461–373).

The mass-to-charge ratios of the major ions in the EI mass spectra of procyclidine and benzhexol are 84/204/105 and 98/218/105, respectively (Table II). Proposed chemical structures of ions consistent with the results are shown in Figures 1 and 2. Because the same corresponding ions are present in the EI mass spectra of the mono-TMS derivative of hydroxyprocyclidine (compound III, Figure 1) and hydroxybenzhexol (compound VII, Figure 2), respectively, metabolic hydroxylation in both parent drugs must have taken place at the cyclohexane ring. In fact, had hydroxylation taken place at the aromatic ring, the formation of the ions of *m/z* 204, 218, and 105 would not have been possible. It was noteworthy that the assignment of the site of metabolic hydroxylation in a xenobiotic molecule has a strong bearing on analytic method development because it may determine the physicochemical properties of the target analyte. In both procyclidine and benzhexol, hydroxylation at the aromatic ring would have resulted in the formation of amphoteric compounds. However, the fact that hydroxylation had taken place at the cyclohexane ring meant that the hydroxy metabolites possessed basic properties that were expected to be as nearly the same as the parent drugs.

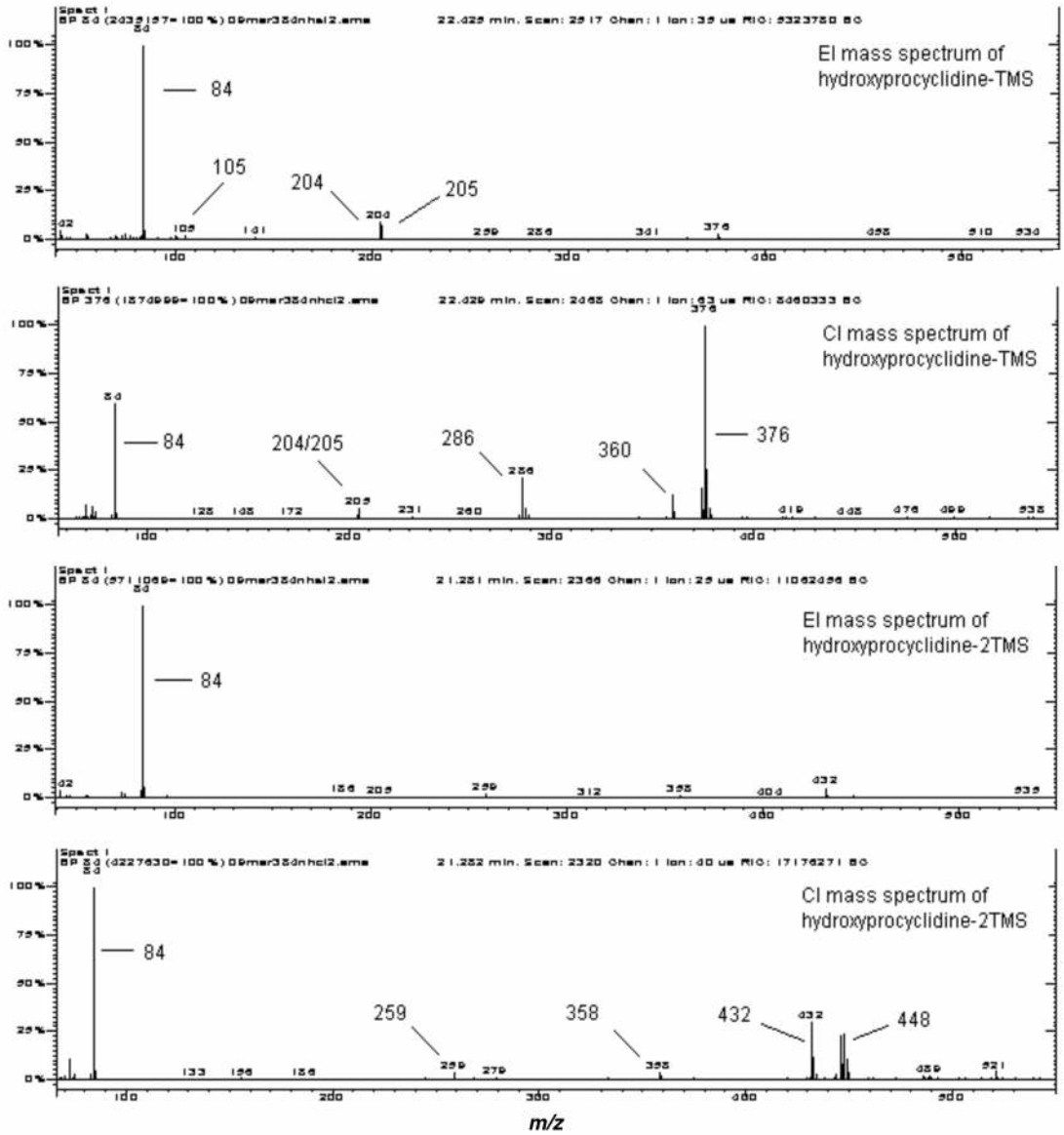

Figure 3. Electron impact and chemical ionization mass spectra of the mono- and di-TMS derivatives of hydroxyprocyclidine obtained on the Varian Saturn GC–MS instrument.

From an analytical perspective, amphoteric compounds require tighter pH-range adjustments for solvent extraction than basic compounds. Moreover, aromatic hydroxy groups are generally more reactive towards glucuronide conjugation than alcoholic hydroxy groups (15,16). Such a situation may have a bearing on the need and extent of β-glucuronidase hydrolysis step in sample preparation for GC–MS analysis, which for the hydroxy metabolites of procyclidine and benzhexol will be considered in the Analytic method optimization subsection.

The difference of 72 between the molecular weights of compounds IV and III for hydroxyprocyclidine (447–375) and compounds VIII and VII for hydroxybenzhexol (461–389) was indicative of introduction of a second TMS group at the intrinsic hydroxy group of the parent drugs and therefore formation of di-TMS derivatives of the hydroxy metabolites. Further evidence of this was obtained by successively increasing the temperature or time of heating with the derivatizing reagent. The peaks due to compounds III and VIII, which were the mono-TMS derivatives of hydroxyprocyclidine and hydroxybenzhexol, respectively, decreased in intensity, whereas those due to compounds IV and IX, which were the di-TMS derivatives of the hydroxy metabolites, increased in intensity with increasing the temperature or time of heating with the derivatizing reagent. It was almost certain that mono-TMS derivatization of the hydroxy metabolites had taken place preferentially at the metabolically introduced secondary hydroxy group because it was less sterically hindered than the intrinsic tertiary one.

Regarding the characterization of the hydroxy metabolites, the mono-TMS derivatives in the EI mass spectra contained substantially more fragment ions than the di-TMS derivatives (Table II) and were, therefore, more structurally informative. In fact, the EI mass spectra of the di-TMS derivatives of hydroxyprocyclidine and hydroxybenzhexol were totally dominated by the methylenepyrrolidinium and methylenepiperidinium base-peak ions, respectively, so that other ions were of very low abundance. On the other hand, the CI mass spectra of both the mono- and di-TMS derivatives exhibited a number of ions of substantial abundance and were, therefore, structurally informative. An added advantage of the CI mass spectra of the di-TMS derivatives is their suitability for use in quantitative analysis for two reasons: attainment of oneness of the target analyte, upon using appropriate experimental conditions, and enhanced sensitivity. Representative EI and CI mass spectra of the mono- and di-TMS derivatives of hydroxyprocyclidine are shown in Figure 3.

Analytic method optimization

Having identified the target urinary analytes for procyclidine and benzhexol, two factors regarding the analytic method optimization remained to be addressed: urine hydrolysis and derivatization. The Agilent GC–EIMS system was employed in the study. By measuring the heights of the chromatographic peaks corresponding to the mono- and di-TMS derivatives of hydroxyprocyclidine and hydroxybenzhexol obtained before and after enzyme hydrolysis (but under otherwise identical experimental conditions) approximate ratios of 1:1 were obtained. Equal recoveries of the hydroxy metabolites of procyclidine and benzhexol, before and after hydrolysis, were thus indicated with two implications. Firstly, the hydroxy metabolites did not undergo phase II metabolic glucuronide conjugation to any significant extent. Secondly, when hydroxyprocyclidine or hydroxybenzhexol is the only target urinary analyte, omission of the hydrolysis step will save on material and time. Cleaner extracts with consequent less congested chromatograms are additional advantages of the omission of the hydrolysis step. However, the two antimuscarinics are usually abused with other drugs, mostly the benzodiazepines, for which sample hydrolysis is an essential step.

The underivatized hydroxy metabolites were not detected under the experimental conditions used due to either thermal degradation and/or none elution from the GC column. Acetyl derivatization was attempted as an alternative to trimethylsilyl derivatization in case a silylating derivatizing reagent was not available; no acetyl derivative of either hydroxy metabolite was detected under the experimental conditions used, possibly due to degradation.

Conclusions

The major target analytes for procyclidine and benzhexol in urine were found to be their cyclohexane-ring mono substituted hydroxy metabolites. Phase II metabolic conjugation of the hydroxy metabolites did not occur. TMS derivatization was found to be a requisite for the detection of the metabolites. Mono- and/or di-TMS derivatives were formed. The yield of either form depended on the temperature and heating time with the derivatizing reagent. Which form to target was governed by the analytical application, be it qualitative, quantitative, or both.

If immunoassays are to be developed for presumptive testing of procyclidine or benzhexol abuse, they should target the hydroxy metabolites rather than the parent drugs.

Despite liquid chromatography–MS–MS taking over in the study of metabolic profiles of xenobiotics, this study shows that GC–MS, in both the EI and CI modes, still holds a firm position in that respect.

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