High resolution mass spectrometry elucidates metabonate (false metabolite) formation from alkylamine drugs during in vitro metabolite profiling

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Abbreviations used are: ESI, Electrospray ionization; FWHM, Full width at half maximum; HDMS, High-definition mass spectrometry; HIM, Human intestinal microsomes; HLM, Human liver microsomes; IDA, Information-dependent acquisition; LC, Liquid chromatography; LC/MS/MS, Liquid chromatography tandem mass spectrometry; LC/MS<sup>E</sup>, Liquid chromatography/elevated energy mass spectrometry; m/z, Mass to charge ratio; MIST, Metabolites in safety testing; MS, Mass spectrometry; MS<sup>E</sup>, Elevated energy mass spectrometry; NA, Not applicable; ND, Not detected; NL, Neutral loss; ToF, Time of flight; UHPLC, Ultra high-performance liquid chromatography.
In vitro metabolite profiling and characterization experiments are widely employed in early drug development to support safety studies. Samples from incubations of investigational drugs with liver microsomes or hepatocytes are commonly analyzed by liquid chromatography/mass spectrometry for detection and structural elucidation of metabolites. Advanced mass spectrometers with accurate mass capabilities are becoming increasingly popular for characterization of drugs and metabolites, spurring changes in the routine workflows applied. In the present study, using a generic full scan high-resolution data acquisition approach with a time-of-flight mass spectrometer combined with post-acquisition data mining, we detected and characterized metabonates (false metabolites) in microsomal incubations of several alkylamine drugs. Had a targeted approach to mass spectrometric detection (without full scan acquisition and appropriate data mining) been employed, the metabonates may not have been detected, hence their formation under-appreciated. In the absence of accurate mass data, the metabonate formation would have been incorrectly characterized since the detected metabonates manifested as direct cyanide-trapped conjugates or as cyanide-trapped metabolites formed from the parent drugs by the addition of 14 Da, the mass shift commonly associated with oxidation to yield a carbonyl. This study demonstrates that high-resolution mass spectrometry and the associated workflow is very useful for the detection and characterization of unpredicted sample components and that accurate mass data were critical to assignment of the correct metabonate structures. Additionally, for drugs containing an alkylamine moiety, the results suggest that multiple negative controls and chemical trapping agents may be necessary to correctly interpret the results of in vitro experiments.
INTRODUCTION

Establishing the metabolic profiles associated with enzymatic biotransformation of new investigational drugs is an integral part of drug development (Bateman et al, 2007; Tiller et al, 2008). Metabolites can potentially contribute to the pharmacological activity and adverse effects of drugs, as evidenced by the interest by regulatory agencies in metabolites in safety testing (MIST) and metabolites as potential perpetrators of drug-drug interactions. Consequently, some understanding of the metabolism of a new drug is desirable prior to Phase 1 clinical studies in humans. To aid selection of appropriate toxicology species as well as the design of appropriate safety studies, metabolite formation is assessed in \textit{in vitro} incubations of drug candidates with liver microsomes, S9 fraction or hepatocytes from humans and nonclinical species (Li et al, 2006; Tiller et al, 2008). These same \textit{in vitro} test systems are commonly employed to assess metabolic clearance of the parent drug (Yin et al, 2001) and to investigate the formation of reactive (electrophilic) metabolites (Park et al, 2011), although the formation of reactive metabolites itself does not necessarily imply toxicity (Prakash et al, 2008).

Liquid chromatography tandem mass spectrometry (LC/MS/MS) is the analytical technique of choice for characterizing metabolites both \textit{in vitro} and \textit{in vivo} due to its sensitivity and selectivity, and for elucidating the structure of detected metabolites. Increasingly, high resolution mass spectrometry (\textit{i.e.} resolving power > 10,000 FWHM) is being employed for metabolite profiling and characterization because accurate mass data establish the elemental composition and fragmentation characteristics of detected drug-related components, which improves structure elucidation and the identification of metabolic soft spots and routes of biotransformation (Sanders et al, 2006; Castro-Perez et al, 2005). Time-of-flight (ToF) mass spectrometers are high-resolution instruments with fast cycle times making them compatible with the higher-resolution ultra-high pressure liquid chromatography (UHPLC) systems currently available (Castro-Perez et al, 2005). The combination of UHPLC with ToF mass spectrometry provides a robust platform for rapid profiling and characterization of metabolites formed from new drugs.
and enables implementation of generic analytical workflows, such as MS\textsuperscript{E} (elevated energy mass spectrometry), comprising simultaneous acquisition of precursor (m/z values corresponding to intact metabolites for biotransformation assignment) and product ion data (tandem mass spectral fragmentation data for structural elucidation of metabolites) by generating full scan mass spectral data which are subsequently deconvoluted and mined by software strategies capitalizing on the high information content associated with accurate mass (Bateman et al, 2007; Wrona et al, 2005). Such generic workflows, also known as qualitative/quantitative analysis, yield complex data sets that can be mined in different ways to obtain different types of information. Since the complete m/z range data set is acquired for each sample, these workflows support the analysis of unknowns making them useful tools for qualitative \emph{a priori} characterization of metabolites. The generic full scan ToF MS\textsuperscript{E} mass spectrometry workflow with post-acquisition data mining has been described in detail for screening for reactive metabolites elsewhere (Barbara and Castro-Perez, 2011).

While \textit{in vitro} drug discovery assays provide essential pharmacology and toxicology information, occasional instances of artifacts have been described. Metabonate formation is an unusual type of artifact that can be extremely difficult to distinguish from enzymatic metabolism. The term metabonate was originally coined by Beckett (1971) to describe a compound formed by the reaction between a metabolite and a chemical (interferent) in the sample matrix. Because it is dependent on metabolite formation, metabonate formation is time-, protein- and cofactor-dependent, with the hallmarks of metabolite formation (Beckett, 1971). Metabonates are neither true metabolites nor simple chemical artifacts.

In the present study, we describe formation of metabonates when several alkylamine-containing drugs are incubated with NADPH-fortified human liver microsomes. This was initially observed in microsomal incubations of the calcium channel blocker diltiazem and its major mono-demethylated metabolite nordiltiazem (Scheme 1), and was confirmed with a mechanistic study involving multiple stable-isotope labeling strategies. Subsequent reactive metabolite screening of additional compounds by
electrophilic trapping revealed similar metabonate formation from at least two additional alkylamine drugs. The full scan high-resolution mass spectrometry analysis with data mining for unknowns was essential to the detection and characterization of the metabonates.
MATERIALS AND METHODS

Materials
Nordiltiazem, dinordiltiazem and all stable-isotope-labeled reagents were purchased from Toronto Research Chemicals (Ontario, Canada). Human liver and intestinal microsomes (HLM and HIM) and high-purity MilliQ water were prepared at XenoTech, LLC (Lenexa, KS). Optima grade acetonitrile was purchased from ThermoFisher Scientific (Waltham, MA). Diltiazem and all other chemicals and reagents were purchased from Sigma-Aldrich (St. Louis, MO).

Metabolite profiling incubations
Diltiazem, nordiltiazem or their deuterated analogs (50 or 100 µM) were incubated individually at 37°C with NADPH-fortified pooled HLM or HIM (1 mg/mL) in potassium phosphate buffer (100 mM, pH 7.4) containing 10 mM MgCl₂, in the presence or absence of glutathione (1 mM), KCN (1 mM), semicarbazide (1 mM) or 30% (v/v) 13C-labeled formaldehyde. The substrates were added in water, as were all other incubation components. Zero-substrate and zero-cofactor samples served as controls. Reactions were terminated at 15 or 30 min by addition of an equal volume of acetonitrile (CH₃CN, C₂H₅CN or 13CH₂-13C15N). Precipitated protein was removed by centrifugation and supernatant fractions were analyzed by LC/MS/MS.

Chemical methylation
Nordiltiazem (100 µM) was dissolved in 100 mM potassium phosphate buffer (pH 7.4) with 30% (v/v) formaldehyde. After 30 min, an equal volume of acetonitrile was added and the sample was analyzed by LC/MS/MS.
**Reactive metabolite screening incubations**

Clozapine, indinavir, ketoconazole, nicotine, nefazodone, phencyclidine, prochlorperazine and ticlopidine (100 μM) were individually incubated at 37°C with NADPH-fortified HLM (1 mg/mL) in potassium phosphate buffer (100 mM, pH 7.4) and KCN (1 mM) for 90 min under the conditions described by Argoti *et al.* (2005). Precipitated protein was removed by centrifugation and supernatant fractions were analyzed by LC/MS/MS.

**Instrumental analysis**

Chromatographic separation was achieved on a Waters Acquity UltraPerformance LC (UHPLC) system (Waters Corp., Milford, MA) with a Waters Acquity HSS T3 column (1.8 μM, 2.1 x 100 mm) heated to 40°C. The mobile phases were 0.1% formic acid in water and 0.1% formic acid in acetonitrile.

*Metabolite profiling incubations*

Following 10 μL injections, a linear gradient from 10 – 80% acetonitrile from 1 to 9 min and then held at 80% acetonitrile for 1 min was employed at a total flow rate of 0.3 mL/min. The UHPLC system was interfaced by electrospray ionization (ESI) to a Waters Synapt G1 High Definition Mass Spectrometer (HDMS), a quadrupole ToF instrument, operated in full scan MS<sup>E</sup> positive V-mode. The MS<sup>E</sup> mode comprises two interleaved full scan functions to obtain accurate mass precursor and product ion data for all components detected with one injection in a data-independent manner. Data were acquired over the range 100 – 1000 m/z using a capillary voltage of 3.2 kV, sampling cone voltage of 40 V, source temperature of 120°C and desolvation temperature of 300°C. For the high energy scan function, a collision energy ramp of 15 – 45 eV was applied at the Trap traveling wave ion guide. A continuous lockspray reference compound was sampled at 10 second intervals for centroid data mass correction.

*Reactive metabolite screening incubations*

Following 5 or 10 μL injections, a 10-minute linear gradient ramping from 2 – 90% acetonitrile was applied at a total flow rate of 0.5 mL/min. The UHPLC system was interfaced by ESI to a Waters
Synapt G2 HDMS operated in full scan MS<sup>E</sup> positive resolution mode. Data were acquired over the mass range 50 – 1200 using a capillary voltage of 2.7 kV, sampling cone voltage of 40 V, source temperature of 120°C and desolvation temperature of 400°C, with a Trap collision energy ramp of 10 – 30 eV and 10-second interval lockmass correction. Additional unit-resolution analysis for the reactive metabolite screening samples was performed with the UHPLC interfaced with ESI to an AB Sciex API 4000 QTrap MS (Foster City, CA) operating in neutral loss 27 (NL27) information-dependent acquisition (IDA) mode for detection of low level cyanide-trapped metabolites. Data were acquired over the ranges 150-450 and 450-750 m/z. The MS instrument was operated with a capillary voltage of 4.0 kV, a cone voltage of 56 V, source temperature at 600°C, and collision energy of 29 eV. The trigger threshold for IDA of product ion spectra was 1000 counts.

**Data processing**

Accurate mass data acquisition was achieved with MassLynx version 4.1 SCN 712 (Waters Corp., Milford, MA). Data were processed with MetaboLynx XS software, a component of MassLynx. Data were initially filtered (± 0.035 amu) according to accurate mass defect based on substrate/conjugate elemental compositions using the structure-based C-heteroatom dealkylation tool (Mortishire-Smith et al, 2009) to construct appropriate mass defect filters for each individual substrate with or without cyanide trapping. Filtered sample and control (zero-cofactor and zero-substrate) data were compared with MetaboLynx XS software to obtain metabolite profiles for the incubated substrates. Unit resolution mass data acquisition was achieved with Analyst version 1.4.2 (AB Sciex, Foster City, CA). Data were processed with LightSight 2.2 software (AB Sciex, Foster City, CA) for sample and control comparison. Spectral interpretation and structural elucidation were performed manually.
RESULTS

Metabolite profiling of diltiazem

Incubation of 50 μM diltiazem with NADPH-fortified HLM and HIM resulted in LC/MS/MS detection of 14 putative NADPH-dependent metabolites (M1-M14). Figure 1 shows ion chromatogram profiles for diltiazem and its metabolites formed by human liver (Fig. 1a) and intestinal microsomes (Fig. 1b) derived by mass defect data filtering (± 35 mDa) of the low energy full scan MS^6 data. The profiling data (relative peak areas of diltiazem) showed more extensive metabolism of diltiazem in HLM than in HIM. M1-M14 were all formed by HLM whereas only 11 of them (all except M3, M11 and M12) were formed by HIM. The relative abundance of all detected metabolites was greater in HLM than in HIM.

Accurate mass data (Table 1) enabled derivation of elemental compositions for M1-M13 with <10 ppm mass accuracy and showed that all metabolites, with the exception of M12, were formed by established routes of biotransformation including hydroxylation, O- or N-demethylation, ester hydrolysis (resulting in de-acetylation), or a combination thereof. These results are consistent with published literature (Molden et al, 2003). The elemental composition search parameters (see Supporting Information) were limited to ±10 ppm and were based on the structure of diltiazem and possible elemental composition changes associated with known routes of microsomal biotransformation. The elemental composition of M12 (C_{20}H_{19}NO_{5}S) and associated elemental shift from diltiazem [-(CH_{3})_{2}NH +O] are consistent with a didesmethylamino diltiazem aldehyde metabolite formed by N-dealkylation which cleaves the entire dimethylamino group and forms an aldehyde on the ethyl chain: R-CH_{2}CH_{2}-N(CH_{3})_{2} \rightarrow R-CH_{2}CHO (M12) + (CH_{3})_{2}NH. The proposed structure of M12 is a novel metabolite of diltiazem but a minor metabolite compared with nordiltiazem and di-nordiltiazem (formed by N-demethylation).

The original elemental composition search parameters yielded no hits for M14, which was detected as a protonated molecule of m/z 440.1656. The following findings identified M14 as a metabonate. Broadening the search parameters to more generic values (see Supporting Information) but
still within the ±10 ppm window yielded four elemental composition hits (Table 2). Three of the hits did not make chemical sense, but the fourth (C_{23}H_{26}N_{3}O_{4}S), which corresponded to the addition of cyanide to diltiazem, was consistent with chemical valency requirements. Mass spectral product ion assignments for diltiazem were proposed on the basis of the high energy accurate MS^E spectrum for the diltiazem reference standard (Fig. 2a). Then structural elucidation was performed for M14 on the basis of the proposed elemental composition and the product ions observed in the corresponding high energy MS^E spectrum for M14 (Fig. 2b) compared with the fragmentation behavior of diltiazem. The product ion detected in both spectra at m/z 370.11 is associated with the fragment of diltiazem remaining after neutral loss of the dimethylamine moiety. Its presence indicates that the structural modifications yielding M14 occur on the dimethylamine group itself. The elemental composition of M14 (derived from the accurate mass data) is consistent with the dominant neutral loss of 27.0118 yielding the fragment of 413.1538 observed in the mass spectrum. Neutral loss of 27.01 is uncommon and is usually associated with a loss of HCN. Since a cyanide diltiazem adduct was unexpected, further studies were undertaken to determine the mechanism of formation of M14.

The metabolonate M14: mechanistic studies

Formation of M14 from diltiazem was dependent on time and NADPH; no M14 was observed in the zero-minute or zero-NADPH control incubations. M14 was also formed when nordiltiazem, was incubated under the same conditions. However, in contrast to formation of M14 from diltiazem, the formation of M14 from nordiltiazem was not dependent on NAPDH. Consequently, N-demethylation was considered the first step in formation of M14 from diltiazem.

Since no cyanide salts were added during the microsomal incubations, the two possible sources of the incorporated cyano group were acetonitrile employed as the organic mobile phase and acetonitrile used as the stop reagent. Changing the mobile phase from acetonitrile to methanol had no effect on M14.
formation. Conversely, stopping the reactions with methanol instead of acetonitrile eliminated M14 formation.

Initially, M14 was proposed as an unusual acetonitrile adduct of nordiltiazem, so incubations were performed with stable-isotope-labeled acetonitrile employed as the stop-reagent. One set of microsomal incubations with diltiazem was stopped with C\textsubscript{2}H\textsubscript{3}CN whereas a duplicate set was stopped with \textsuperscript{13}CH\textsubscript{3}\textsuperscript{13}C\textsubscript{15}N. Formation of M14 occurred in both incubations. The low energy MS\textsuperscript{E} accurate mass spectrum for M14 formed in the incubations stopped with C\textsubscript{2}H\textsubscript{3}CN (Fig. 3a) showed no evidence of incorporation of deuterium. In contrast, the low energy MS\textsuperscript{E} accurate mass spectrum for M14 formed in the incubations stopped with \textsuperscript{13}CH\textsubscript{3}\textsuperscript{13}C\textsubscript{15}N (Fig. 3b) showed that the \textit{m/z} of the protonated molecule had shifted by +1.9984, consistent with incorporation of \textsuperscript{13}C\textsubscript{15}N from the acetonitrile stop reagent. The observed neutral loss of 29.0114, corresponding to the product ion of \textit{m/z} 413.1563, was associated with a neutral loss of H\textsuperscript{15}C\textsubscript{13}N. These studies with isotopically labeled acetonitrile established that the acetonitrile stop reagent is the source of the cyano group incorporated during M14 formation, but acetonitrile is not the source of the additional methylene group incorporated during M14 formation from nordiltiazem.

The possibility of a reactive intermediate involved in the formation of M14 was considered. Accordingly, microsomal incubations of diltiazem were performed in the presence of glutathione, sodium cyanide or semicarbazide in an effort to trap intermediates in M14 formation. The effect of the trapping agents on M14 formation was determined by estimation of the relative LC/MS\textsuperscript{E} peak areas for M14 derived from the low energy extracted accurate mass ion chromatograms for 440.1644±0.035. Peak areas were normalized to M14 formed in a control incubation performed in the absence of a chemical trap (Fig. 4). The addition of cyanide anion increased M14 formation, as expected from the results with isotopically labeled acetonitrile. The addition of glutathione reduced the extent of M14 formation, but no glutathione-conjugated metabolites were detected. The addition of semicarbazide, however, almost abolished
formation of M14, implicating aldehyde involvement in M14 formation. No additional trapped metabolites were detected in any of the incubations.

Formaldehyde has been shown to react with primary and secondary amine groups in a classic Mannich reaction to form an iminium ion (Thompson, 1968). Consequently, another set of microsomal diltiazem incubations was prepared where incubations proceeded in the presence of 30% v/v H\textsuperscript{13}CHO. M14 formation under these conditions was confirmed by LC/MS\textsuperscript{E} analysis, and the accurate mass product ion spectrum associated with the M14 formed in the presence of the stable-isotope-labeled formaldehyde (Fig. 5) showed the protonated molecule detected at \textit{m/z} 441.1674. The M14 formed under these conditions had incorporated the heavy carbon isotope, implicating formaldehyde as the source of the methylene group. This was unexpected because no methanol solvent, which has been described in the literature as a source of formaldehyde in microsomal incubations (Chauret et al, 1998; Yin et al, 2001) was employed in any of the previous incubations. Substrates were dissolved in water.

The results suggested that M14 was formed by cytochrome P450-mediated \textit{N}-demethylation of diltiazem to the secondary amine nordiltiazem which subsequently reacted with formaldehyde (a product of the \textit{N}-demethylation reaction) to yield an iminium ion that reacted with cyanide anion in the acetonitrile stop reagent. To confirm this hypothesis, nordiltiazem was incubated in potassium phosphate buffer with 30% v/v formaldehyde. After 30 min, an equal volume of acetonitrile was added and the sample analyzed by LC/MS. M14 was formed under these conditions, with identical chromatographic retention and mass spectral characteristics to those observed for M14 formed in the original microsomal incubations. The low energy MS\textsuperscript{E} accurate mass spectrum for the reaction product (Fig. 6) showed the protonated molecule detected at \textit{m/z} 440.1659 with sodium and potassium adducts at \textit{m/z} 462.1480 and 478.1753 and the expected product ions at \textit{m/z} 413.1547 and 370.1309 corresponding to the neutral losses of HCN and the modified methylamine group, respectively.
Metabonate formation in reactive metabolite screening

Since the M14 metabonate was observed to form under reactive metabolite screening conditions where diltiazem was incubated in the presence of 1 mM NaCN, additional amine-containing drugs were incubated with HLM in the presence of cyanide anion to determine whether this behavior was confined to diltiazem or prevalent in other structurally-related compounds, in some cases incorporating alkylamine functional groups other than methylamine. These incubation samples were analyzed by the high-resolution LC/MS<sup>E</sup> approach and by LC and the more sensitive neutral loss 27 scan type on a triple-quadrupole linear ion trap because reactive metabolites often form at low abundance so detection can be challenging. Consequently, accurate mass data were acquired for some but not all of the detected cyanide conjugates while unit resolution mass spectral data were obtained for all detected cyanide-trapped species. With cyanide as the trapping agent, at least two cyanide conjugates were detected for each of clozapine, indinavir, ketoconazole, nefazodone, nicotine and prochlorperazine (Table 3). Five conjugates (CL1-CL5) were detected for clozapine, two for indinavir (IN1 and IN2), two for ketoconazole (KE1 and KE2), eight for nefazodone (NE1-NE8), two for nicotine (NI1 and NI2) and seven for prochlorperazine (PR1-PR7). Of these twenty-six detected cyanide conjugates, thirteen were formed by expected oxidative metabolism, eight appeared to be direct cyanide conjugates of the drugs, four (IN1, IN2, NE1 and NE2) were formed through a combination of events including methylenation, and one (PR5) was a conjugate formed by a structural modification associated with the addition of 14 amu and cyanide conjugation. According to the elemental compositions derived from accurate mass data, formation of IN1, IN2, NE1 and NE2 involved N-dealkylation at the piperazine ring of indinavir or nefazodone yielding a secondary amine group, as well as methylenation and cyanide conjugation. Having established that secondary alkylamines are susceptible to reaction with formaldehyde, and since NADPH-fortified liver microsomes lack the cofactor and enzymes that catalyze methylation, these four conjugates are proposed as additional metabonates formed through the mechanism determined for diltiazem. This indicates that the phenomenon is not limited to methylamines but affects the wider class of alkylamine compounds. PR5 may be an additional methylenated metabonate, but this conjugate was not detected by the high-resolution mass spectrometer.
so this possibility cannot be supported with accurate mass data as the mass shift of +14 Da could potentially be associated with oxidation to yield a carbonyl group (+O –H₂). In addition, three of the compounds investigated in the reactive metabolite screening assay, namely clozapine, nicotine and prochlorperazine, contain tertiary methylamine moieties, and apparent direct cyanide conjugates of all three were detected. Metabonate M14 manifested with an elemental composition consistent with a direct cyanide conjugate of diltiazem because its formation involved a combination of –CH₂ and +CH₂ demonstrating that any methylamine-containing compound detected as an apparent direct conjugate could potentially be formed by the metabonate formation mechanism described for diltiazem. Consequently, five other cyanide conjugates (CL4, NE6, NE8, NI1 and NI2) represent additional potential metabonates.
DISCUSSION

In the present study, we have described a two-step process leading to metabonate formation from alkylamine-containing drugs incubated with NADPH-fortified microsomes: the first step involves formaldehyde methylation of the alkylamine to form an iminium ion (by the Mannich reaction between formaldehyde and a primary/secondary amine); the second step involves the reaction of the iminium ion with cyanide anion. Metabonate formation from tertiary amines was NADPH-dependent because enzymatic dealkylation to yield the primary or secondary amine precursor was necessary to precipitate the reaction with formaldehyde, as demonstrated with diltiazem, a dimethylamine, and nordiltiazem, a monomethylamine (Fig. 6).

Metabonate formation involved the incorporation of CH₂ (from formaldehyde) and CN (from acetonitrile). Metabonate formation from diltiazem occurred only after N-demethylation (loss of CH₂) to nordiltiazem. Accordingly, metabonate formation from diltiazem (+24.9964 Da) appeared to involve only the addition of cyanide (+CN [26.0031] –H [1.0078]) because the methylation reaction (+CH₂) effectively canceled the mass change associated with N-demethylation.

The first reports of metabonate formation coined the term metabonate to describe an experimental artifact dependent on enzymatic biotransformation (Beckett, 1971). It is not synonymous with a simple artifact produced solely by a chemical reaction. For the drugs tested in our study, metabolism by N-dealkylation preceded metabonate formation. Analogously, the metabolates reported by Beckett (1971) were also formed from alkylamine compounds, predominantly amphetamines, and the first step in their formation was enzymatic N-dealkylation. Subsequent intramolecular cyclization between the secondary amine (the N-dealkylation product) and an accessible carbonyl group formed the detected metabonates. A similar phenomenon was later reported for N-benzyl-4-chloroaniline, again initiated by N-dealkylation, this time at the center of the molecule to yield a primary amine and an aldehyde which subsequently reacted to form the Schiff base, namely N-benzylidene-4-chloroaniline, confirmed with a chemical
standard analyzed by NMR spectroscopy (Low et al, 1994). Metabonate formation from N-benzyl-4-chloroaniline occurs by a process consistent with our proposed mechanism of metabonate formation.

Organic solvents can have undesirable consequences such as decreased enzymatic activity in in vitro drug metabolism studies conducted with hepatocytes and subcellular fractions. Consequently, organic solvent use is minimized where possible in microsomal and hepatocyte incubations, although methanol and acetonitrile are considered less problematic than ethanol and acetone (Chauret et al, 1998). Methanol is oxidized to formaldehyde in hepatocyte and microsomal incubations; the formaldehyde produced has been shown to react with primary and secondary amine drugs yielding artifacts formed by methylenation and cyclization (Yin et al, 2001). Although metabolism of the incubated drugs did not contribute to the metabonate formation described, the artifacts were technically metabonates since their formation involved enzymatic metabolism of methanol. The described metabonate formation resulted in errors in microsomal stability estimates made on the basis of substrate loss over time. Yin et al. (2001) reported that the concentration of formaldehyde formed from methanol metabolism reached up to 600 μM over 60 min. In a second report describing formation of similar methylene artifacts from 1,2-ethylene diamine-containing compounds, the source of the aldehyde component was again proposed as alcohol solvent, established through experiments with ethanol, methanol and deuterium-labeled methanol (Li et al, 2006). However, alcohol solvents were not employed in the present study so methanol was not the source of the formaldehyde supporting the methylenation reaction. The formaldehyde came either from N-demethylation reactions or from an unidentified source.

Glycerol is used to stabilize microsomal preparations, and it can be metabolized by NADPH-fortified microsomes to an aldehyde consistent with formaldehyde (Clejan and Cederbaum, 1992). Artifact formation from acyl glucuronides as a direct result of the presence of glycerol in human liver S9 fraction has been reported (Obach, 2009). However, the generation of aldehydes as by-products of N-dealkylation reactions in the absence of glycerol or methanol is unavoidable; in the case of methylamines, N-demethylation will necessarily produce formaldehyde. Analogously, heteroatom demethylation of any endogenous substrates in the microsomal incubation will also lead to formaldehyde formation.
Gorrod and colleagues (1994) reported the formation of artifacts and metabonates from amine-containing drugs under \textit{in vitro} reactive metabolite screening conditions (\textit{i.e.} in the presence of an electrophilic trapping reagent). Initially, cyanide trapping with cyanide salts in the incubation matrix was established as a stabilization technique for the detection of reactive iminium intermediates formed from alicyclic amines such as nicotine (Gorrod and Aislatner, 1994), because iminium ions react with cyanide anion to form a stable nitrile product (Gorrod and Sai, 1997). Secondary alicyclic amine compounds were subsequently shown to undergo a methylenation reaction with formaldehyde to yield a compound stabilized by reaction with cyanide anion. The described artifacts were proposed to form by the Mannich reaction to yield iminium ion intermediates stabilized with cyanide to cyanomethyl compounds. Analogous to our work, the presence of an excess of the aldehyde trapping agent semicarbazide inhibited formation of the artifacts, while co-incubation of the alicyclic amine drugs with compounds known to produce formaldehyde during their metabolism increased metabonate formation (Gorrod and Sai, 1997). These results provide further support to our proposed mechanism of metabonate formation from alkylamine compounds. It is noteworthy that the investigators observed formaldehyde generation upon cofactor addition to fresh microsomes in the absence of organic solvent which may implicate demethylation of endogenous substrates as a source of formaldehyde, as suggested above.

Our initial experiments were conducted in the absence of cyanide salts because metabolite profiling was the goal, but we detected cyanide conjugates nevertheless. When the incubations were repeated in the presence of an excess of cyanide anion, metabonate formation increased significantly, as expected. Through experiments with stable-isotope labeled acetonitrile we established the source of the cyano group in the metabolite profiling experiments as cyanide anion present in the acetonitrile stop reagent. Interestingly, incubation in an excess of glutathione trapping agent did not yield any glutathione adducts, but partially inhibited metabonate formation. Based on a recent publication describing the reaction of glutathione with formaldehyde (Hopkinson et al, 2010), we propose that glutathione may compete with the incubated drug to react with formaldehyde, effectively scavenging the aldehyde although not as efficiently as semicarbazide, which eliminated metabonate formation.
Recently, a report on cyanide-trapped metabonate formation under reactive metabolite screening incubation conditions was published (Rousu and Tolonen, 2011). Consistent with our findings, the authors described structural changes to amine moieties of three drugs (propranolol, amlodipine and ciprofloxacin), resulting in the addition of a CH₂ group and cyano conjugation, and ascribed these structural changes to metabonate formation. This study provides further support for our supposition that the metabonate formation issue may be applicable to multiple amine-containing drugs and drug candidates and demonstrates the need for mass spectrometric detection as opposed to analysis by techniques that yield no structural information about detected components, e.g., UV/radiometric detection. However, although the authors state that a Mannich reaction may be involved in the formation of an iminium ion stabilized by the addition of cyanide anion, their proposed mechanism differs from ours. They proposed that cytochrome P450-mediated hydroxylation or dehydrogenation reactions are the initial NADPH-dependent step in metabonate formation. This initial step in their proposed mechanism for propranolol would yield a tertiary amine incapable of undergoing the proposed Mannich reaction with formaldehyde as a primary or secondary amine is required (March, 1992). In light of this and our mechanistic studies, combined with the aforementioned literature reports, we propose that metabonate formation from amine-containing drugs occurs by our proposed dealkylation, formaldehyde methylenation and cyanide conjugation mechanism.

We have described the phenomenon of in vitro metabonate formation from amine-containing drugs for metabolite profiling and reactive metabolite screening studies, and proposed a mechanism based on results derived from accurate MS analysis, stable-isotope labeling experiments and wet chemistry techniques. The described phenomenon explains unexpected results by our group (Barbara et al, 2010) as well as other research groups (Rousu and Tolonen, 2011). The detected species were detected by us and by Rousu et al. because we employed an accurate mass full scan technique for metabolite profiling. In reactive metabolite screening studies employing the NL 27 scan type for cyanide-conjugate detection, these metabonates would have been detected, and may explain the unassigned cyanide conjugates formed from amine-containing drugs detected by Argoti et al. (2005). With conventional triple quadrupole and
quadrupole ion trap techniques such as targeted multiple reaction monitoring or neutral loss/precursor ion scan types, analogous metabonates may not have been detected and their structures and routes of formation would have been mis-assigned. Consequently, this problem may be considerably widespread. While similar issues have been reported in microsomal stability work, multiple studies where conclusions are drawn from substrate loss or metabolite formation data such as reaction phenotyping and enzyme kinetic investigations could also be affected. Accurate mass spectrometry analysis was essential to identification of the metabonates because with unit resolution the mass shift associated with metabonate formation could have been interpreted as the result of oxidation with formation of carbonyl metabolites accompanied by cyanide trapping. Consequently, in the absence of accurate mass spectral data, in vitro LC/MS/MS metabolite profiles and reactive metabolite screening results for alkylamine compounds could be misinterpreted, potentially compromising the development of drug candidates.

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AUTHORSHIP CONTRIBUTIONS

Participated in research design: Barbara, Toren, Parkinson

Conducted experiments: Barbara, Kazmi, Muranjan

Contributed new reagents or analytic tools: Barbara, Muranjan, Toren

Performed data analysis: Barbara, Muranjan, Parkinson

Wrote or contributed to the writing of the manuscript: Barbara, Parkinson
REFERENCES


FOOTNOTES:

Part of this work was presented previously: Barbara JE, Kazmi F, Toren PC and Parkinson A (2010) Accurate mass spectrometry elucidates a misleading metabolite formed from amine-containing drugs in reactive metabolite screening assays. Ninth ISSX Meeting; 2010 Sept 4-8; Istanbul, Turkey. International Society for the Study of Xenobiotics, Washington, DC.
FIGURE LEGENDS

Scheme 1: Cytochrome P450-mediated conversion of diltiazem to its N-demethylated metabolites nordiltiazem and di-nordiltiazem with accurate mass and elemental composition data

Fig. 1: Representative mass defect filtered (±35 mDa) ion chromatograms showing the metabolite profile for 50 μM diltiazem incubated with NADPH-fortified human (a) liver microsomes and (b) intestinal microsomes (1 mg/mL)

Fig. 2: High energy product ion mass spectra for (a) diltiazem reference standard and (b) M14 formed from diltiazem (50 μM) incubated with NADPH-fortified human liver microsomes (1 mg/mL) with proposed structures and fragment assignments (inset)

Fig. 3: Low energy full scan mass spectra for M14 formed from diltiazem (100 μM) incubated for 30 min with NADPH-fortified human liver microsomes (1 mg/mL) stopped with (a) C2H3CN and (b) 13CH313C15N with proposed structures and fragment assignments (inset)

Fig. 4: Relative formation of M14 in incubations of diltiazem (100 μM) with NADPH-fortified human liver microsomes (1 mg/mL) for 30 min in the presence of the trapping agents glutathione, cyanide ion and semicarbazide. Values are normalized to M14 formation in the absence of trapping agent

Fig. 5: Quadrupole precursor ion selection product ion mass spectrum for M14 formed from diltiazem (100 μM) incubated for 30 min with NADPH-fortified human liver microsomes (1 mg/mL) in the presence of 30% 13C-formaldehyde with proposed structure and fragment assignments (inset)

Fig. 6: Low energy full scan mass spectrum for M14 formed from nordiltiazem (100 μM) incubated for 30 min in potassium phosphate buffer with 30% formaldehyde with proposed structures and fragment
assignments (inset)

Fig. 7: Proposed scheme of metabonate formation from diltiazem and nordiltiazem in microsomal incubations stopped with acetonitrile
Table 1. Diltiazem-related components detected by high-resolution mass spectrometry following incubation of 50 μM diltiazem with NADPH-fortified human liver and intestinal microsomes (1 mg/mL) for 30 min

<table>
<thead>
<tr>
<th>Identity</th>
<th>Retention Time (min)</th>
<th>Experimental m/z</th>
<th>Mass shift from diltiazem</th>
<th>Proposed elemental composition</th>
<th>Proposed metabolite</th>
<th>Mass error (ppm)</th>
<th>HLM</th>
<th>HIM</th>
</tr>
</thead>
<tbody>
<tr>
<td>M1</td>
<td>5.14</td>
<td>431.1661</td>
<td>15.9969</td>
<td>C_{22}H_{26}N_{2}O_{5}S</td>
<td>Hydroxy diltiazem</td>
<td>4.6</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>M2</td>
<td>5.63</td>
<td>359.1455</td>
<td>-56.0237</td>
<td>C_{19}H_{23}N_{3}O_{5}S</td>
<td>O-desmethyl desacetyl diltiazem</td>
<td>7.2</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>M3</td>
<td>5.57</td>
<td>417.1507</td>
<td>1.9815</td>
<td>C_{21}H_{24}N_{2}O_{5}S</td>
<td>Hydroxy O-desmethyl diltiazem</td>
<td>5.5</td>
<td>+</td>
<td>ND</td>
</tr>
<tr>
<td>M4</td>
<td>6.04</td>
<td>401.1564</td>
<td>-14.0128</td>
<td>C_{21}H_{24}N_{2}O_{4}S</td>
<td>O-desmethyl diltiazem</td>
<td>7.2</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>M5</td>
<td>6.12</td>
<td>387.1408</td>
<td>-28.0284</td>
<td>C_{20}H_{22}N_{2}O_{4}S</td>
<td>O-desmethyl nordiltiazem</td>
<td>7.5</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>M6</td>
<td>6.33</td>
<td>431.1649</td>
<td>15.9957</td>
<td>C_{22}H_{26}N_{2}O_{5}S</td>
<td>Hydroxy diltiazem</td>
<td>1.9</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>M7</td>
<td>6.41</td>
<td>373.1615</td>
<td>-42.0077</td>
<td>C_{20}H_{22}N_{2}O_{4}S</td>
<td>Desacetyl diltiazem</td>
<td>7.8</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>M8</td>
<td>6.55</td>
<td>359.1461</td>
<td>-56.0231</td>
<td>C_{19}H_{22}N_{2}O_{5}S</td>
<td>Desacetyl nordiltiazem</td>
<td>8.9</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>M9</td>
<td>6.97</td>
<td>415.1715</td>
<td>0.0023</td>
<td>C_{22}H_{26}N_{2}O_{5}S</td>
<td>NA</td>
<td>5.5</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>M10</td>
<td>7.04</td>
<td>401.1563</td>
<td>-14.0129</td>
<td>C_{21}H_{24}N_{2}O_{4}S</td>
<td>Nordiltiazem</td>
<td>7.0</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>M11</td>
<td>7.07</td>
<td>387.1389</td>
<td>-28.0303</td>
<td>C_{20}H_{22}N_{2}O_{4}S</td>
<td>Di-nordiltiazem</td>
<td>2.6</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>M12</td>
<td>7.44</td>
<td>403.1365</td>
<td>-12.0327</td>
<td>C_{20}H_{22}N_{2}O_{4}S</td>
<td>Hydroxy di-nordiltiazem</td>
<td>9.2</td>
<td>+</td>
<td>ND</td>
</tr>
<tr>
<td>M13</td>
<td>8.63</td>
<td>417.1494</td>
<td>1.9802</td>
<td>C_{21}H_{24}N_{2}O_{5}S</td>
<td>Hydroxy nordiltiazem</td>
<td>2.4</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>M14</td>
<td>9.34</td>
<td>440.1656</td>
<td>+24.9964</td>
<td>No hits</td>
<td>Unknown</td>
<td>NA</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

+ Detected  ND Not detected  NA Not applicable

HLM Human liver microsomes  HIM Human intestinal microsomes
Table 2. Elemental composition hits for m/z 440.1656 (M14) based on broad generic search parameters within 10 ppm accurate mass error listed in order of isotope pattern fitting

<table>
<thead>
<tr>
<th>Elemental composition</th>
<th>Theoretical m/z</th>
<th>Mass error (ppm)</th>
<th>Elemental composition shift from diltiazem</th>
<th>iFit</th>
</tr>
</thead>
<tbody>
<tr>
<td>C_{28}H_{26}NO_{2}S</td>
<td>440.1684</td>
<td>-6.4</td>
<td>+6C -N -2O</td>
<td>48.6</td>
</tr>
<tr>
<td>C_{26}H_{24}N_{4}OS</td>
<td>440.1671</td>
<td>-3.4</td>
<td>+4C +2N -2H -3O</td>
<td>48.9</td>
</tr>
<tr>
<td>C_{23}H_{26}N_{3}O_{4}S</td>
<td>440.1644</td>
<td>2.7</td>
<td>+C +N</td>
<td>49.3</td>
</tr>
<tr>
<td>C_{29}H_{26}N_{2}O</td>
<td>440.1637</td>
<td>4.3</td>
<td>+7C +2N -3H -3O -S</td>
<td>50.4</td>
</tr>
</tbody>
</table>
Table 3. Cyanide-trapped reactive metabolites detected by high-resolution mass spectrometry following incubation of multiple substrates (100 μM) with NADPH-fortified human liver microsomes (1 mg/mL) for 90 min in the presence of NaCN (1 mM)

<table>
<thead>
<tr>
<th>Drug</th>
<th>Conjugate</th>
<th>Unit resolution m/z</th>
<th>Proposed composition</th>
<th>Accurate m/z</th>
<th>Mass error (ppm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Clozapine</td>
<td>CL1</td>
<td>334</td>
<td>P+CN+O-Cl</td>
<td>334.1656</td>
<td>-3.5</td>
</tr>
<tr>
<td></td>
<td>CL2</td>
<td>368</td>
<td>P+CN+O</td>
<td>368.1275</td>
<td>-0.8</td>
</tr>
<tr>
<td></td>
<td>CL3</td>
<td>368</td>
<td>P+CN+O</td>
<td>368.1275</td>
<td>-0.8</td>
</tr>
<tr>
<td></td>
<td>*CL4</td>
<td>352</td>
<td>P+CN</td>
<td>352.1328</td>
<td>-0.2</td>
</tr>
<tr>
<td></td>
<td>CL5</td>
<td>338</td>
<td>P+CN-CH₂</td>
<td>338.1160</td>
<td>-3.6</td>
</tr>
<tr>
<td>Indinavir</td>
<td>**IN1</td>
<td>578</td>
<td>P+dealkylation+O+CN+CH₂</td>
<td>578.3350</td>
<td>2.2</td>
</tr>
<tr>
<td></td>
<td>**IN2</td>
<td>562</td>
<td>P+dealkylation+CN+CH₂</td>
<td>562.3402</td>
<td>2.5</td>
</tr>
<tr>
<td>Ketoconazole</td>
<td>KE1</td>
<td>572</td>
<td>P+CN+O</td>
<td>ND</td>
<td>NA</td>
</tr>
<tr>
<td></td>
<td>KE2</td>
<td>556</td>
<td>P+CN</td>
<td>556.1481</td>
<td>-6.7</td>
</tr>
<tr>
<td>Nefazodone</td>
<td>**NE1</td>
<td>415</td>
<td>P+dealkylation+O+CN+CH₂</td>
<td>415.2452</td>
<td>0.0</td>
</tr>
<tr>
<td></td>
<td>**NE2</td>
<td>399</td>
<td>P+dealkylation+CN+CH₂</td>
<td>399.2505</td>
<td>0.5</td>
</tr>
<tr>
<td></td>
<td>NE3</td>
<td>527</td>
<td>P+CN+2O</td>
<td>ND</td>
<td>NA</td>
</tr>
<tr>
<td></td>
<td>NE4</td>
<td>511</td>
<td>P+CN+O</td>
<td>ND</td>
<td>NA</td>
</tr>
<tr>
<td></td>
<td>NE5</td>
<td>511</td>
<td>P+CN+O</td>
<td>ND</td>
<td>NA</td>
</tr>
<tr>
<td></td>
<td>NE6</td>
<td>495</td>
<td>P+CN</td>
<td>ND</td>
<td>NA</td>
</tr>
<tr>
<td></td>
<td>NE7</td>
<td>511</td>
<td>P+CN+O</td>
<td>ND</td>
<td>NA</td>
</tr>
<tr>
<td></td>
<td>NE8</td>
<td>495</td>
<td>P+CN</td>
<td>ND</td>
<td>NA</td>
</tr>
<tr>
<td>Nicotine</td>
<td>*NI1</td>
<td>188</td>
<td>P+CN</td>
<td>188.1184</td>
<td>-1.9</td>
</tr>
<tr>
<td></td>
<td>*NI2</td>
<td>188</td>
<td>P+CN</td>
<td>188.1184</td>
<td>-1.9</td>
</tr>
<tr>
<td>Prochlorperazine</td>
<td>PR1</td>
<td>415</td>
<td>P+CN+O</td>
<td>415.1361</td>
<td>0.5</td>
</tr>
<tr>
<td>------------------</td>
<td>------</td>
<td>------</td>
<td>--------</td>
<td>----------</td>
<td>------</td>
</tr>
<tr>
<td>PR2</td>
<td>431</td>
<td>P+CN+2O</td>
<td>431.1313</td>
<td>0.9</td>
<td></td>
</tr>
<tr>
<td>PR3</td>
<td>415</td>
<td>P+CN+O</td>
<td>ND</td>
<td>NA</td>
<td></td>
</tr>
<tr>
<td>*PR4</td>
<td>399</td>
<td>P+CN</td>
<td>399.1410</td>
<td>0.0</td>
<td></td>
</tr>
<tr>
<td>*PR5</td>
<td>399</td>
<td>P+CN+14</td>
<td>ND</td>
<td>NA</td>
<td></td>
</tr>
<tr>
<td>PR6</td>
<td>429</td>
<td>P+CN+2O</td>
<td>ND</td>
<td>NA</td>
<td></td>
</tr>
<tr>
<td>*PR7</td>
<td>399</td>
<td>P+CN</td>
<td>ND</td>
<td>NA</td>
<td></td>
</tr>
</tbody>
</table>

*Potential metabonate

**Metabonate

ND Not detected
NA Not applicable
Scheme 1

Diltiazem
\[ \text{C}_{22}\text{H}_{26}\text{N}_{2}\text{O}_{4}\text{S} \]
\[ [M + H]^+_{\text{th}} = 415.1692 \]

CYP
N-Demethylation

\[ \text{O}_2 + \text{NADPH}_2 \]
\[ \rightarrow \text{HCHO} + \text{H}_2\text{O} + \text{NADP} \]

Nordiltiazem
\[ \text{C}_{21}\text{H}_{24}\text{N}_{2}\text{O}_{4}\text{S} \]
\[ [M + H]^+_{\text{th}} = 401.1535 \]

CYP
N-Demethylation

\[ \text{O}_2 + \text{NADPH}_2 \]
\[ \rightarrow \text{HCHO} + \text{H}_2\text{O} + \text{NADP} \]

Di-nordiltiazem
\[ \text{C}_{20}\text{H}_{22}\text{N}_{2}\text{O}_{4}\text{S} \]
\[ [M + H]^+_{\text{th}} = 387.1379 \]
Figure 1

(a)

(b)

Unlabeled peaks are not related to diltiazem.
Figure 2

(a)

(b)
Figure 3

(a)

No heavy isotopes present

(b)

$^{13}$C and $^{15}$N present
Figure 4
Figure 5

$\text{CH}_3$

$R - N^{13} \text{CH}_2C\equiv N$

$^{12}\text{C} \text{ present}$

$[M+H]^+$

441.1674

$^{13}\text{C} \text{ absent}$

$[M-\text{HCN}+H]^+$

414.1566

415.1869

$^{13}\text{C} \text{ present}$

$[M+\text{Na}]^+$

463.1512

464.1607

$\text{m/z}$
Figure 6
Figure 7

\[
\begin{align*}
\text{CH}_3 & \quad \text{R-N} \quad \text{N-Demethylation} \\
& \quad \text{CYP} \\
& \quad \text{O}_2 + \text{NADPH}_2 \quad \rightarrow \\
& \quad \text{HCHO} + \text{H}_2\text{O} + \text{NADP} \\
\text{CH}_3 & \quad \text{R-NH} \quad \text{Detected} \\
& \quad \text{Chemical} \quad \text{(Schiff base)} \\
\text{HCHO} & \quad \text{H}_2\text{O} \\
& \quad \text{Detected} \\
\text{R-N}^+ \text{CH}_2 & \quad \text{Chemical} \quad \text{(Unknown)} \\
& \quad \text{CN}^- \\
& \quad {^2}\text{H}_3\text{CN} \quad {^{13}}\text{CH}_3{^{13}}\text{C}^{15}\text{N} \\
\text{CH}_3 & \quad \text{R-N-CH}_2\text{C}==\text{N} \\
& \quad \text{Detected}
\end{align*}
\]