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Application of semi-automated metabolite identification software in the drug discovery process for rapid identification of metabolites and the cytochrome P450 enzymes responsible for their formation

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Abstract

Rapid identification of metabolites of compound X using data dependent scan function of a quadrupole ion trap mass spectrometer and semi-automated metabolite identification software is described. Compound X is metabolized via monooxygenation and desmethylation. LC-ESI-MS spectra obtained, following incubations of Compound X with microsomes in the presence and absence of chemical inhibitors specific for CYP1A2, CYP3A4, CYP2D6, CYP2C9 and CYP2E1, were processed using semi-automated metabolite identification software to extract information and to identify the cytochrome P450 enzymes responsible for metabolite formation. Chemical inhibition data suggests that the primary cytochrome P450 (CYP450) isozyme responsible for the metabolism of compound X is CYP3A4 with a minor contribution from both CYP2D6 and CYP2E1. Additionally, neither CYP2C9 nor CYP1A2 appears to contribute to the metabolism of compound X.

Keywords: Drug metabolism; Chemical inhibition; Automated metabolite identification software; LC-MS/MS; Drug discovery; Structure elucidation; CYP450

1. Introduction

In order to improve the number of successful drug candidates in the pre-clinical, clinical and commercial stages of drug development, the drug discovery process is constantly scrutinized and improved [1]. Adding to this pressure is the generation of the vast number of new chemical entities through the use of combinatorial chemistry technologies [2]. To accommodate this pressure on the pharmaceutical industry, the role of drug metabolism has changed in the drug discovery process [3]. Specifically, early metabolite identification information can be helpful to the discovery or medicinal chemist to block some of the

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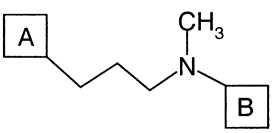
metabolic 'hot spots' and produce an 'ideal' drug that is less susceptible to metabolism [4,5]. In addition, it is preferred that the 'ideal' drug be metabolized by several cytochrome P450 (CYP450) isozymes to minimize the potential for drug–drug interactions (induction and inhibition) [6]. Thus, it is necessary in the drug discovery stage to identify compounds that are metabolized by a single isozyme and those compounds, which are mainly metabolized by polymorphic enzymes. Currently, chemical inhibition data, using isoform-selective inhibitors of cytochrome P450, are obtained either by using radiolabeled parent drug or by monitoring the change in parent drug concentration in a bioanalytical assay [7–9]. Unfortunately, these methods fail to give any information about the biotransformation of the compound or aid in developing the 'ideal' drug [10].

In this paper, we have demonstrated the application of a semi-automated metabolite identification software and the data dependent scan function of an ion trap mass spectrometer [11] for rapid identification of metabolites of compound X and for identifying the CYP450 isozymes responsible for metabolite formation through the use of isoform selective chemical inhibitors of cytochrome P450 [12].



Compound X

2. Experimental

2.1. Sample preparation

Compound X (4.55 μM, dissolved in 25% DMSO/75% ACN) was incubated with human liver microsomes (0.455 mg/ml, purchased from GENTEST Corporation, Woburm, MA) at 37 °C with 0.9 mM NADPH in the presence and absence of known inhibitors of CYP450 (9.1 μM furafylline, 4.6 μM quinidine, 9.1 μM sulfaphenazole, 90.9 μM troleandomycin and 45.5 μM diethyldithiocarbamate). The final DMSO and organic concentrations were less than 0.03% and 0.2% respectively [13]. The substrate concentration was selected to match that of the physiological concentration and the concentration of each of the inhibitors was selected based on reported values [14]. All incubation mixtures were pre-incubated for 3 min at 37 °C and the reaction initiated by addition of the inhibitor followed immediately by addition of compound X. For incubations with mechanism-based inactivators (furafylline, troleandomycin and diethyldithiocarbamate), microsomes, NADPH and inhibitor were pre-incubated for 15 min. The reaction was then initiated with addition of compound X. Appropriate control incubations were also included and all incubations were carried out in duplicate. One milliliter sample aliquots were removed at 0–60 min and quenched with 2 ml of acetonitrile. The samples were extracted for 10 min on a vibrax shaker followed by centrifugation at 4 °C for 10 min at 3000 rpm. The supernatant was transferred to fresh tubes and the pellet extracted with another 2 ml acetonitrile. The combined supernatants were evaporated to dryness under a stream of nitrogen. All samples were reconstituted in the same volume of mobile phase prior to analysis by LC-MS.

2.2. LC-MS

All LC-MS and LC-MS/MS experiments were performed using a quadrupole ion trap (LCQ-Deca) mass spectrometer (ThermoFinnigan, San Jose, CA) coupled with HPLC and UV detection. Components of the HPLC and UV detector included the following: HP 1100 Binary Pump with Degasser, autosampler and a multi-wavelength UV detector (Agilent Technologies, Wilmington, DE). The mobile phase consisted of 25 mM ammonium acetate with the pH adjusted to 4.0 with acetic acid (solvent A) and acetonitrile (solvent B). The flow rate was maintained at 1.0 ml/min with a gradient elution of the parent drug (compound X) and metabolites

being achieved on a Phenomenex Aqua C-18 (4.6 × 150 mm) column with a Metasil AQ guard column. A programmed linear gradient started at 15% B and increased to 75% B over 40 min and returned to 15% B for 5 min to equilibrate the column. The HPLC effluent was directly infused into the UV detector set at 276 nm. The effluent was then split such that 25% was introduced into the mass spectrometer via the supplied ESI interface and the remaining 75% diverted to the radioactive detector or to the waste. The mass spectrometer was operated in the positive mode and the source voltage, capillary voltage and tube lens offset were maintained at 4500, 3 and −5 V, respectively. The heated capillary was set at 260 °C to aid with desolvation. Nitrogen was used as the sheath and auxiliary gas and supplied at 80 and 20 units, respectively. Full scan mass spectra were obtained over the mass range of 100–1000 Da. Helium was used for CID at a power of 36% to obtain all MS/MS (MS^2) data. The precursor ion isolation width during MS/MS experiments was set at 1 amu.

3. Results and discussion

Metabolite screening was achieved using the Metabolite ID (ThermoFinnigan, San Jose, CA) software. The molecular weight of compound X was entered and the software was set-up to look for possible metabolites by adding or subtracting nominal mass differences associated with common phase I metabolic modifications. The raw data file for screening was created by subtracting the raw data file of the 0 min incubate from that of the 60 min incubate. The results suggested that compound X underwent monooxygenation and desmethylation. The sites of modification were elucidated using data dependent LC-MS/MS experiments (Figs. 1–4). Using the LC-MS conditions described, a reference standard of compound X eluted at 35.7 min and yielded molecular ion ($[M+H]^+$) at m/z of 383. Under the MS/MS conditions used, $[M+H]^+$ ions of m/z 383 fragmented to give ions at m/z 216, 168, 148, 137 and 109, where m/z 216, 109 and 137 correspond, respectivley to B^+ and A–CH_2^+ and

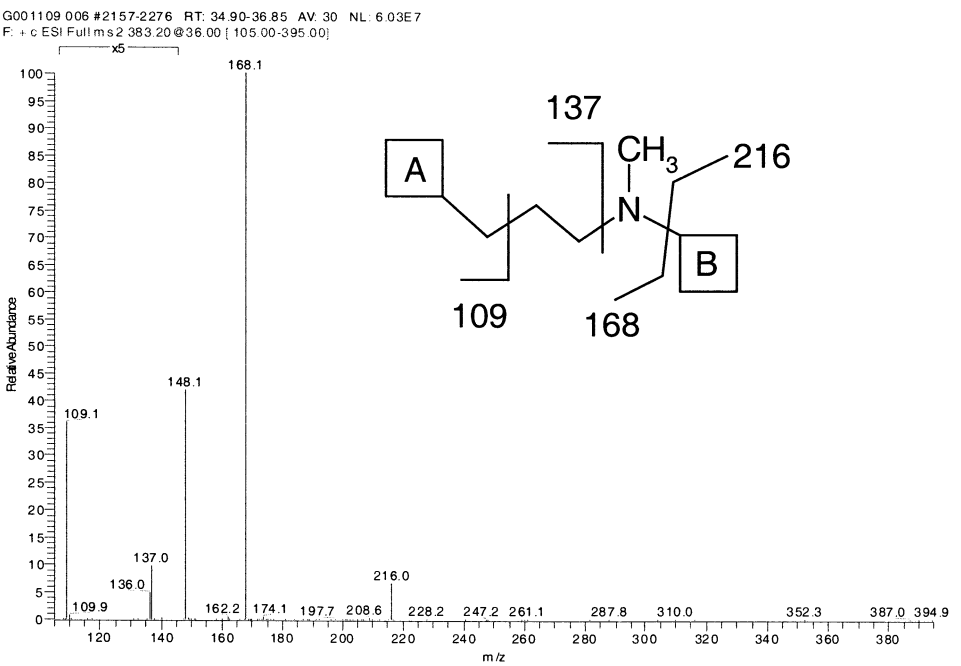


Fig. 1. LC-MS/MS spectrum of compound X.

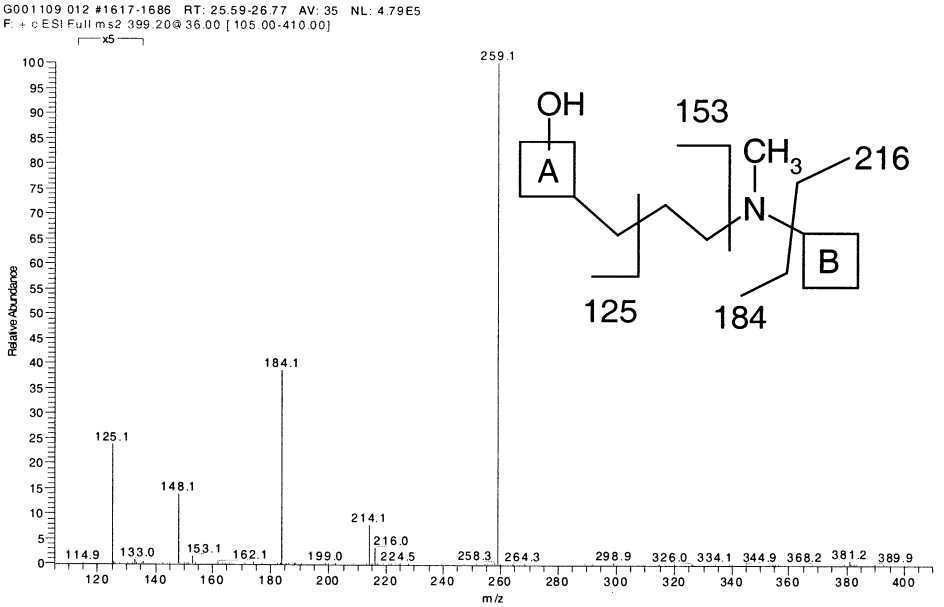


Fig. 2. LC-MS/MS spectrum of metabolite M_1.

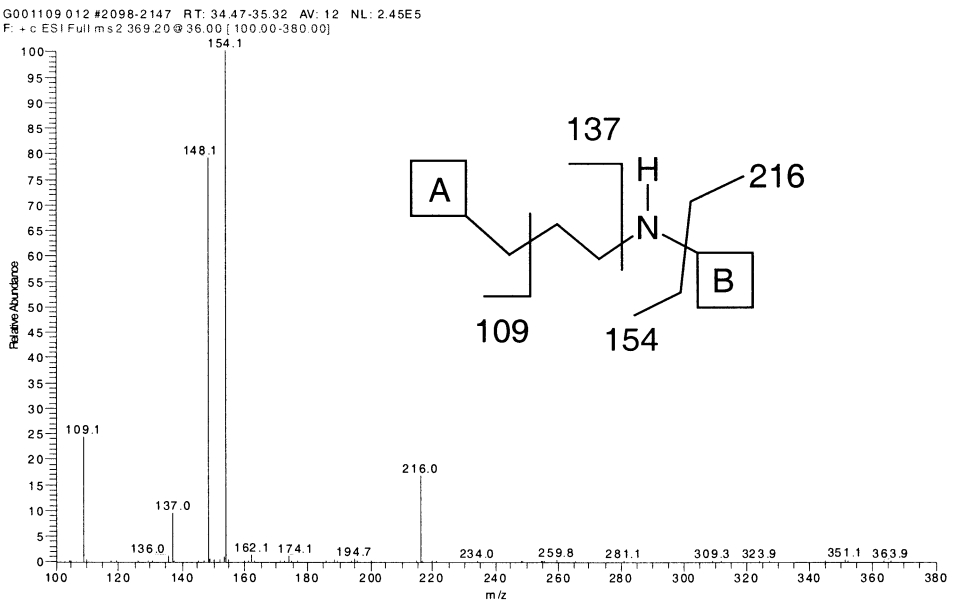


Fig. 3. LC-MS/MS spectrum of metabolite M_2.

A–CH_2–CH_2–CH_2^+(Fig. 1). The fragment ion at m/z 168 is associated with A–CH_2–CH_2–CH_2–NH_2^+–CH_3 and m/z 148 is formed through some rearrangements involving moiety B that will not be discussed in this paper due to proprietary reasons.

Present in the 0 min control incubate was compound X while the 60 min incubation contained

unchanged drug and metabolites M_1, M_2 and M_3 (Figs. 5 and 6). Under the HPLC conditions used, metabolite M_1 eluted at 26.1 min and showed a molecular ion at m/z 399. A molecular weight of 398 corresponding to an increase of 16 Da over the molecular weight of compound X (382) suggests modification by addition of an oxygen atom. A 16 Da shift of the unchanged compound X product ions of m/z 109, 137 and 168 to corresponding product ions of m/z 125, 153 and 184 clearly indicate that oxygenation is occurring on the A–CH_2^+ moiety (Fig. 2). The product ion of m/z 216 suggests that the B moiety of compound X is not modified. Because there is no possibility of forming an N-oxide on this portion of the molecule, M_1 was labeled as a monohydroxylated metabolite.

Metabolite M_2 eluted at 34.6 min and showed a molecular ion at m/z 369. A molecular weight of 368, corresponding to a decrease of 14 amu over the molecular weight of compound X suggests that the metabolite of m/z 369 is most likely formed through desmethylation. Comparison of

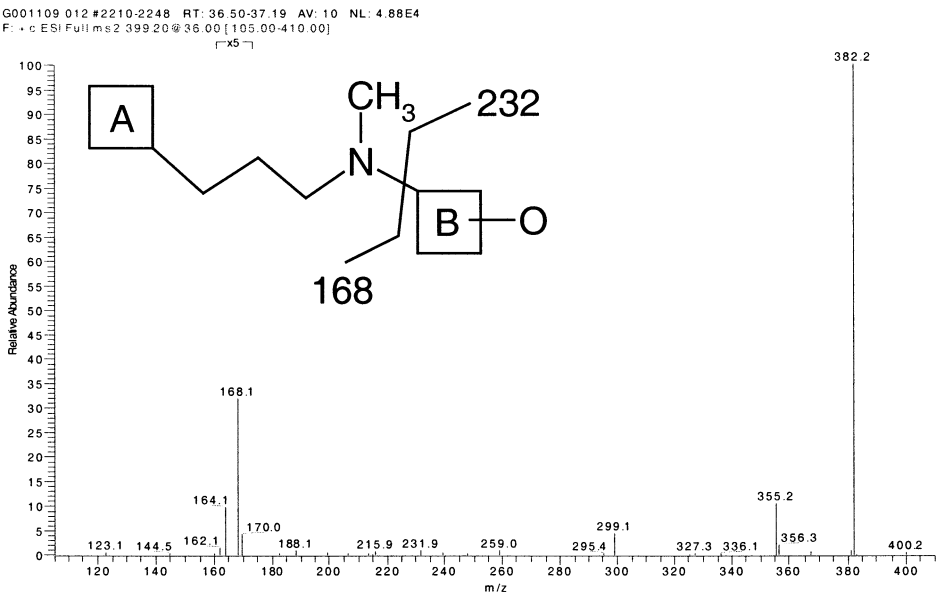


Fig. 4. LC-MS/MS spectrum of metabolite M_3.

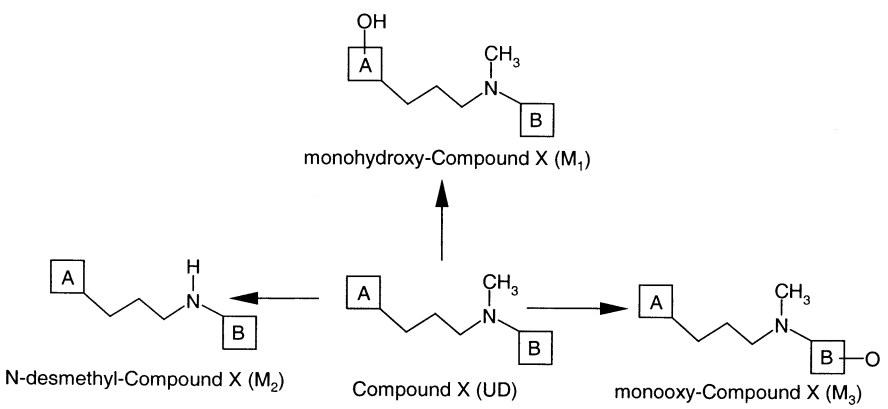


Fig. 5. Proposed biotransformation pathway for compound X.

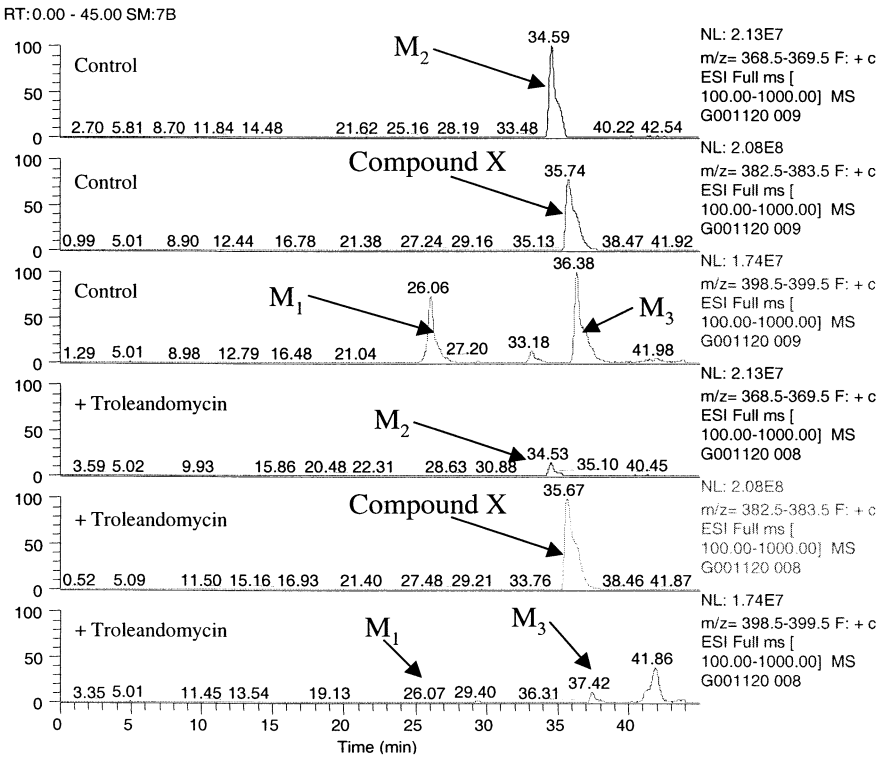


Fig. 6. Extracted ion chromatograms for M_1, M_2, M_3 and compound X in the presence and absence of Troleandomycin, a CYP3A4 inhibitor.

the product ion spectrum for M_2 with that of compound X reference standard suggests that the A–CH_2^+ and B moieties are intact. A 14 Da shift of the unchanged compound X product ion of m/z 168 to corresponding product ion of m/z 154 clearly indicates that desmethylation is occurring on the aminomethyl portion of compound X (Fig. 3). Thus confirming that M_2 is a N-desmethylated metabolite of compound X.

The third metabolite, eluting at 36.4 min, was associated with m/z 399. A molecular weight of 398, corresponding to an increase of 16 amu over the molecular weight of compound X suggests that the ion of m/z 399 is most likely from a hydroxylated or an N-oxide metabolite of compound X. The elution order of this peak suggested that it might be an N-oxide metabolite. A 16 amu shift of the unchanged compound X product ion of m/z 216 (Fig. 4) to corresponding product ion of m/z 232 clearly indicates N-oxidation or hydroxylation of the B moiety.

In a second experiment, the LC-MS peak area of each metabolite identified, as shown in the proposed biotransformation pathway (Fig. 5), was integrated from the extracted ion chromatogram and compared to the peak area of the appropriate control following a 60 min incubation of compound X with microsomes, in the presence and absence of isoform selective inhibitors of CYP 450 (sulfaphenazole for CYP2C9, quinidine for CYP2D6, furafylline for CYP1A2, troleandomycin for CYP3A4 and Diethyldithiocarbamate for CYP2E1). To determine which enzymes are responsible for forming the metabolites, all extracted ion chromatograms corresponding to the metabolites and the unchanged drug were normalized to the peak intensities observed for the control incubates. As shown in Fig. 6, following incubation with troleandomycin about 10, 5 and 20% of M_1, M_2 and M_3 were present, respectively. Unlabelled chromatographic peaks (i.e.

Table 1
List of metabolites and the CYP450 enzymes responsible for their formation

Metabolite label	Chemical name	$[M+H]^+$	Approximate % of control				
			CYP2C9	CYP2D6	CYP1A2	CYP3A4	CYP2E1
M_1	Monohydroxy-compound X	399.2	100	70	100	10	90
M_2	*N*-desmethyl-compound X	369.2	100	70	100	5	70
M_3	Monooxy-compound X	399.2	100	80	100	20	90

38.18 and 41.86 min) were determined not to be drug derived based on MS/MS analysis (spectra not shown). A complete analysis indicated that the primary P450 isozyme responsible for the metabolism of compound X was CYP3A4 with a minor contribution from both CYP2D6 and CYP2E1 (Table 1). Additionally, neither CYP2C9 nor CYP1A2 appears to contribute to the metabolism of compound X.

4. Conclusions

The metabolites of compound X were rapidly identified through the combined use of semi-automated metabolite identification software and LC-MS/MS. The major P450 isoform that metabolizes compound X was identified as CYP3A4 without the use of radiolabeled compound or bioanalytical assay. A minor contribution from both CYP2D6 and CYP2E1 was also observed. Clearly, neither CYP2C9 nor CYP1A2 contributed to the metabolism of compound X. These data led us to conclude that by the combined use of data dependent scan function of an ion-trap and semi-automated metabolite identification software, metabolites and the CYP450 isozymes responsible for their formation can be identified during the early drug discovery stage of drug development, thus enabling pharmaceutical companies to prevent late-stage failures due to drug–drug interactions.

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