Journal of Chromatography B, 877 (2009) 4171–4174

Contents lists available at ScienceDirect

Journal of Chromatography B

journal homepage: www.elsevier.com/locate/chromb

Short communication

Rapid determination of tacrine and other drug metabolites in microsomal incubate by newly developed targeting algorithm on UHPLC/TOFMS

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ARTICLE INFO

Article history:
Received 24 August 2009
Accepted 1 November 2009
Available online 6 November 2009

Keywords:
High-throughput
UHPLC/TOFMS
Tacrine
Targeting algorithm
Cytochrome P450

ABSTRACT

A rapid simultaneous determination method for *in vitro* Cytochrome P450 (CYP) activity assay of 1,2,3,4-tetrahydroacridin-9-amine (tacrine) metabolites using ultra high performance liquid chromatography (UHPLC) coupled with computer-assisted in-source collision induced dissociation (CID) monitoring was investigated. In general, enzyme inhibition assays require quantitative analysis of incubates with drugs using various concentrations of substrates/inhibitors. The assay of CYP isozyme inhibition is an important informational step in the drug discovery process and, with the many substrates listed by the FDA, high-throughput qualitative and quantitative analyses are desired. Based on sub-2-micron packing material with reversed phase chromatography combined with a single liquid chromatography/time-of-flight mass spectrometry (LC/TOFMS), a less than 1 min analysis time is presented for two additional drugs. We successfully determined seven of the eight potential isomer metabolites for the drug tacrine in 2.5 min using a 2 mm internal diameter × 100 mm length column and applying in-source CID with our newly developed chromatographic peak deconvolution technique. Although two of the peaks were heavily fused at a peak width of less than 300 ms, we could clearly identify these peaks by monitoring the chromatographic intensity difference of their fragment peaks on the mass spectrum.

1. Introduction

Recent progress of ultra high performance liquid chromatography (UHPLC) using sub-2-micron packing material drastically improves analysis cycle time on chromatography which makes it possible to separate more components within 1 min while increasing sensitivity [1]. A single liquid chromatography/time-of-flight mass spectrometry (LC/TOFMS) offers the advantage of monitoring all analytes on all chromatographic domains: monitoring multiple compound elutions on a fused chromatographic elution profile by using simultaneous chromatography peak detection rather than tandem mass spectrometry which monitors a single precursor ion at a time.

However, even with chromatographic conditions carefully optimized, it is difficult to separate all the compounds from the target analyte. Using simultaneous chromatographic peak detection and determined target compounds as a ratio of molecular ion chromatogram and in-source collision induced dissociation (CID), a detection method is presented which allows one to obtain purified high-resolution mass spectra from fused chromatographic peaks. Hidden unknown components are clarified by simple software operations.

Biller and Biemann introduced a simple method in which the extracted spectrum is composed of all of the mass spectral peaks that maximize simultaneously [2]. Colby improved the resolution of this method by computing more precise ion maximization times [3]. Stein reported extended a published method of "model peak" approach [4]. The above methods essentially find chromatographic peaks constructed from a series of centroided mass spectra. In the case of liquid chromatography, more components co-elute and ion chromatogram intensity for the target compounds are not strong enough compared to the background components. This may lead to targeted peaks becoming hidden. In addition to this, ion chromatograms consisting of centroided spectra could ignore important impurity components, which have less than the instruments resolving power m/z difference due to the previous centroid process. TIC-based chromatographic peak detection may detect a peak with enough intensity, but many targeted metabolite peaks are difficult to detect. We have tried a new method based on multiple narrow m/z mass chromatograms calculated from the LC/TOFMS profile spectra in order to find target compound with increase detection sensitivity.

This detection method could play an important role in the drug discovery process. Drug–drug interaction studies are an important step in the drug discovery process as they serve as a screening mechanism for interactions that occur in metabolic

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doi:10.1016/j.jchromb.2009.11.003

pathways so that subsequent *in vivo* testing is unnecessary (Food and Drug Administration. Guidance for Industry: Drug Interaction Studies—Study Design, Data Analysis, and Implications for Dosing and Labeling, 2006). They involve Cytochrome P450 (CYP) kinetic studies, which require a large number of qualitative and quantitative *in vitro* analyses of metabolites [5]. We have developed a new compound targeting algorithm based on chromatography peak deconvolution that allows qualitative and quantitative analysis using single LC/TOFMS data with CID. We have evaluated the applicability of this method in CYP activity assays for the drugs 1,2,3,4-tetrahydroacridin-9-amine (tacrine), *N*-(4-ethoxyphenyl)ethanamide (phenacetin), and *N*-[(butylamino)carbonyl]-4-methylbenzenesulfonamide (tolbutamide).

The majority of our study focused on tacrine which is a drug commonly used as a treatment for Alzheimer's disease [5]. Although several papers report on analyzing tacrine metabolites using chromatography, they mostly report on the major metabolites, 1-, 2- and 4-hydroxytacrine [6]. In addition to this, analytical throughput is not high enough to perform a kinetic study.

The tacrine metabolites are not commercially available and can be generated using CYP enzymes or organic chemical synthesis. Accurate mass chromatogram monitoring based on our newly designed targeting algorithm was effective in quickly determining possible metabolite peaks from a complex sample matrix. This technique allows a single LC/TOFMS to become an instrument that can be used for quantitative high-throughput analysis.

2. Materials and methods

2.1. Materials

All chemicals were purchased from Sigma–Aldrich Chemical Co. (St. Louis, MO, US). NADPH Regenerating System Solutions A and B were purchased from BD Biosciences (Bedford, MA, US). ESI-L Low Concentration Tuning Mix was purchased from Agilent Technologies (Santa Clara, CA, US). Bovine liver was purchased from the local grocery store and store at −80 °C until use.

2.2. Ultracentrifuge

Beckman–Coulter Model Optima MAX-XP Ultracentrifuge with an MLA-80 rotor was used.

2.3. HPLC

Agilent Model 1200 series Binary Pump SL and CTC Model HTC-PAL equipped with sample stack cooling unit were used. A YMC-UltraHT Pro18, 2 mm internal diameter × 100 mm length column was used. Flow rate was set to 0.5 mL/min. An 85:15 isocratic elution of water and acetonitrile both containing 0.3% formic acid was used.

2.4. Mass spectrometer

JEOL Model JMS-T100 (AccuTOF) was used after in-house modifications made to improve chromatography retention time reproducibility and data acquisition speed. The modified AccuTOF consistently achieved a resolving power of 10,000 at m/z 609. Based on in-house system suitability tests using four sulfa drugs the overall retention time reproducibility was about %CV = 0.05 (n = 600) and the average mass accuracy was less than 1 ppm using ESI-L Low Concentration Tuning Mix as a lock mass sample.

Data acquisition and data processing software was newly developed.

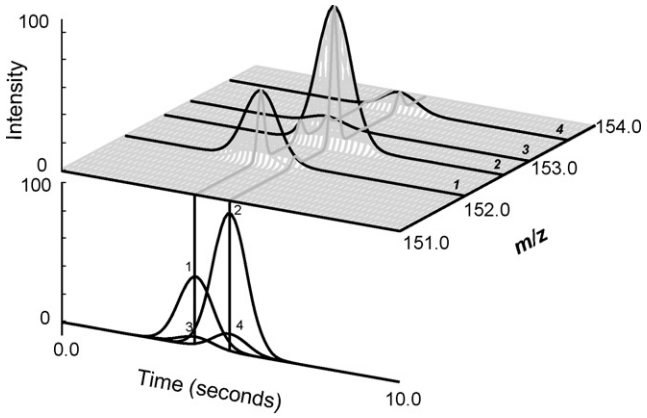

Fig. 1. Example of the peak identification algorithm used in Phase 2 of the targeting algorithm. Example shows the ideal peak containing four peaks each with a different m/z. Peaks 1 and 3 have the same retention time as do peaks 2 and 4, therefore the algorithm concludes that there are two different components.

2.5. Preparation of microsomal fractions

Diced bovine liver, 6.037 g, was homogenized in a glass–glass homogenizer with 30 mL of 100 mM phosphate buffer pH 7.4. The homogenized suspension was centrifuged at 10,000 × g for 30 min. The supernatant fraction was centrifuged at 200,000 × g for 1 h. The precipitate was rinsed three times at 200,000 × g and 0.285 g of precipitate was obtained. The final precipitate was suspended into 1.425 mL of 100 mM phosphate buffer pH 7.4. This suspension was split into 100 μL portions and stored at −80 °C until use.

2.6. CYP activity assay procedure

Solutions of 10 mM CYP substrates (tacrine, phenacetin, and tolbutamide) were made by dissolving in 100 mM phosphate buffer pH 7.4. The 100 μL reaction mixture contained 20 μL of appropriately diluted microsomal fraction, 19 μL BD solution A, 1 μL BD solution B, and 6 mM substrate. The reaction was carried out at 37 °C for 2 h and was stopped by adding 100 μL of methanol containing 4 μg/mL of sulfadimethoxine as an internal standard.

2.7. Targeting algorithm

The targeting algorithm consists of three separate phases.

2.7.1. Phase 1

The algorithm takes a user generated list of chemical formulae, specified ion charge state and user identified potential adducts and generates an accurate profile mass chromatogram for all m/z possibilities. In order to increase chromatography peak detection sensitivity, each chromatogram is calculated based on ideal mass spectral peak width calculated from a resolving power of 10,000. For each targeted m/z, seven chromatograms are calculated so that three times the ideal mass spectral peak width range is covered. This avoids the effects of instrument drift, which can create a significant calculation error if left unaccounted for.

2.7.2. Phase 2

The algorithm compares all detected chromatographic peaks and selects the peaks that have the same elution profile. All detected chromatographic peaks are sorted by retention time. All combination of peaks is compared and if more than two peaks are identified as the same peak, they are counted as a single candidate compound (Fig. 1). This is determined by retention time agreement, peak width and tailing factor. A simplified list of candidate peaks is made and, using these peaks, mass chromatograms are recalculated. After the

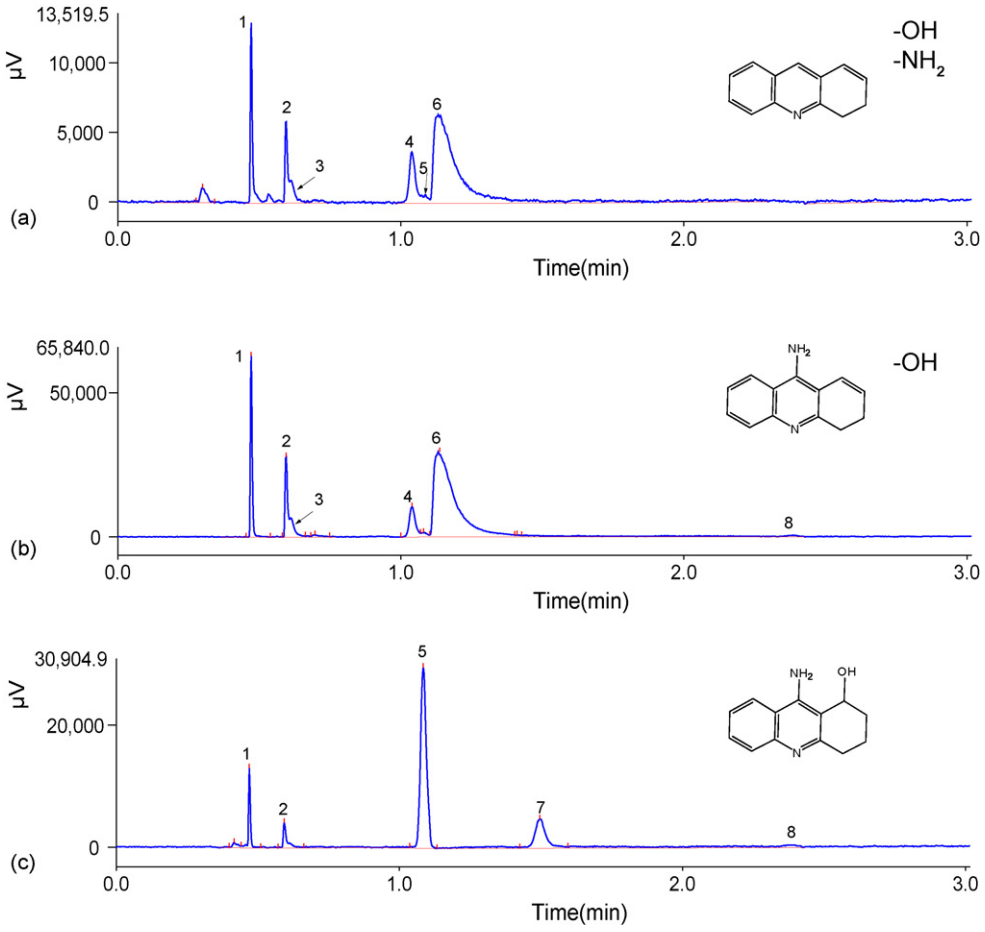

Fig. 2. Chromatogram of microsomal incubate monitored at (a) *m*/*z* 182.097, (b) *m*/*z* 197.108 and (c) *m*/*z* 215.197 obtained from a single sample injection.

chromatographic peaks are detected, a mass spectrum is obtained and the peaks are centroided by selecting the peak that is closest to the targeted *m*/*z* and isotope cluster match.

2.7.3. Phase 3

The algorithm compares elution profiles for the mass chromatograms for all detected mass peaks for each candidate determined by Phase 2. Once this is complete, each spectrum is compared with the isotope cluster profile calculated from the user specified formula. Both chromatographically identified mass peaks and isotope cluster profile matched peaks are graphically represented by a different color.

3. Results and discussion

Rainville et al. wrote that an increased number of new chemical entities (NCEs) are entering the drug discovery and development process. This increased number of compounds requiring analysis means that there is a need for rapid accurate methods. Further, as pharmaceutical companies attempt to make decisions earlier in the drug discovery process, the faster the information can be generated and reviewed the faster decisions can be made in regards to the forward movement of NCEs in the drug pipeline [1]. Drug interaction studies are essentially CYP enzyme inhibition studies that require enzyme reaction velocity analyses, which increases the number of samples to be analyzed. Because of this, the rapid identification of metabolites with a quantitative analysis method is desired.

3.1. Identification of tacrine metabolites (hydroxytacrine)

Microsomal incubate samples were analyzed and no chromatographic peaks at *m*/*z* 215.118 were determined in the control (data not shown). Five peaks at *m*/*z* 215.118 were detected from the sample incubated with 10 μL of microsomal fraction. A metabolite isomer co-elution was suspected for the peak at retention time 0.594 min due to the shoulder at the rear portion of the chromatographic peak (Fig. 2).

Because hydroxytacrine standards are not commercially available, we confirmed the presence of the hydroxytacrine isomers by monitoring the fragment ions created by increasing the orifice 1 voltage from 30 to 90 V. As shown in Fig. 2a and b,

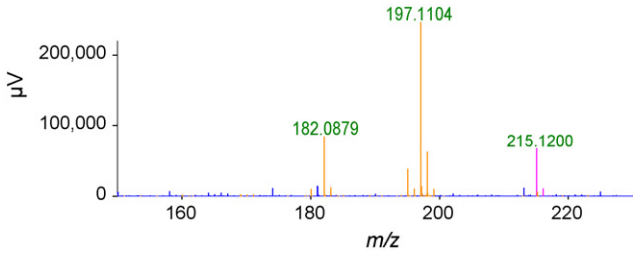

Fig. 3. Example of a mass spectrum from the peak at retention time 0.593 min explaining the algorithm used in Phase 3. The targeted compound is at *m*/*z* 215.12 and appears as magenta because it matches both the targeted compound and isotope cluster match. The peaks at *m*/*z* 182.08 and *m*/*z* 197.11 appear as orange because, while they were not targeted, they have the same elution profile as the peak at *m*/*z* 215.12.

Table 1
Retention time reproducibility for tacrine and hydroxytacrine peaks. Peak identification is as follows: Peaks 1–5 and 7–8 = hydroxytacrine while Peak 6 = tacrine.

Peak #	Average retention time $n = 8$ (min)	Standard deviation (min)	RSD
1	0.47	0.00205	0.0044
2	0.594	0.00280	0.0047
3	0.613	0.00333	0.0054
4	1.024	0.0189	0.0184
5	1.066	0.0213	0.0200
6	1.124	0.0260	0.0231
7	1.468	0.0332	0.0226
8	2.338	0.0596	0.0255

the chromatograms at *m*/*z* 182.097 and *m*/*z* 197.108 have the same chromatographic peaks with different intensity ratios. Our targeting software automatically identified from the retention time analysis that the chromatographic peaks for *m*/*z* 215.118 (Fig. 2c) matched with the chromatographic peaks for *m*/*z* 197.108 (hydroxytacrine–OH) and *m*/*z* 182.097 (hydroxytacrine–OH and $-NH_2$). This is indicated by a color scheme on the mass spectrum—compounds with the same elution profile appear in orange on the mass chromatogram while those with an isotope cluster match appear as red. If both elution profile and isotope cluster match applies to the compound, the mass peak appears as magenta. All other peaks appear as blue and can be discarded as background peaks (Fig. 3).

3.2. Hydroxytacrine isomer identification results

As shown in Table 1, seven hydroxytacrine isomers were identified with retention time reproducibility RSD of 0.4–2.2%. Although the first three peaks were not fully separated, good retention time reproducibility was obtained by monitoring in-source CID chromatograms.

3.3. Accuracy of CYP activity assay

The linearity of several hydroxytacrine peaks according to varying amounts of microsomal fraction was calculated and excellent linear relationship was obtained (data not shown). The results show that this method monitors the maximum velocity of hydroxytacrine enzymatic production and renders it an applicable method for determining drug metabolic reaction inhibition.

3.4. Other metabolite screening

A qualitative experiment was run to determine if we could screen for other CYP metabolites. For the two additional substrates used, phenacetin and tolbutamide, all metabolites were detected by the newly developed targeting algorithm. Isotope matching was used to confirm the targeted compounds were indeed the compounds expected (Fig. 4).

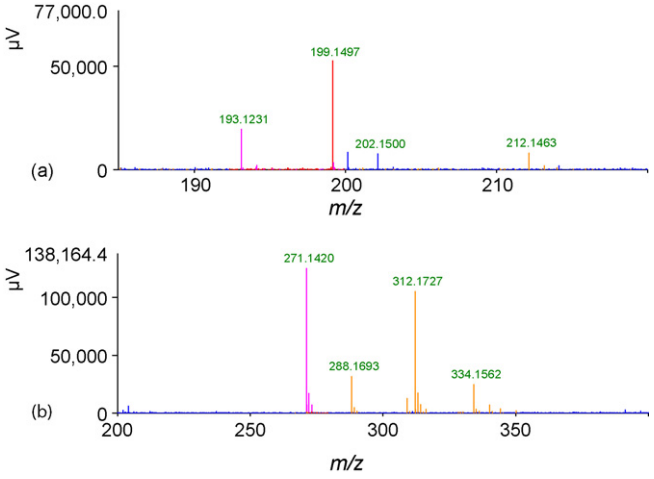

Fig. 4. (a) Spectral peaks for acetaminophen, the CYP assay metabolite for phenacetin and (b) spectral peaks for hydroxytolbutamide, the CYP assay metabolite for tolbutamide.

4. Conclusions

We have demonstrated the rapid determination of tacrine and other drug metabolites using UHPLC performance separation. By comparing chromatographic peak parameters such as retention time, peak width and tailing factor, our algorithm effectively shows all mass peaks that belong to a single component on a chromatogram. The obtained peak height and/or area has a linear relationship to the amount of microsomes added making our quantitative method applicable for enzyme inhibition kinetic studies. In addition, our newly developed targeting algorithm was able to clarify fragments and other peak adducts even when they were not specifically targeted. This is helpful in reviewing large numbers of acquired data and preparing reports in a high-throughput analysis laboratory.

Acknowledgements

The authors would like to thank Sierra Analytics, Inc. for providing their SAToolKit software, Katherine M. Schroeder for preparing the bovine liver microsomal fractions, and Tania B. Hermis for fully validating the software and for preparing all the figures for publication.

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