Identification of Isomeric Phase II Drug Metabolites From Mouse Thin Tissue Sections using Liquid-Extraction Based Surface Sampling Mass Spectrometry

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OVERVIEW

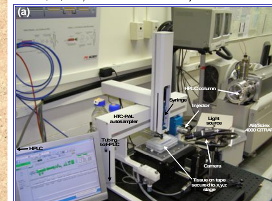
- **GOAL:**
  - Implement a separation step into the liquid extraction-based surface sampling process.
  - Separate and detect metabolite isomers from a mouse thin tissue section that was not achieved before by any known surface sampling method.
- **METHOD:**
  - A commercially available autosampler was adapted to perform direct liquid microjunction (LMJ) surface sampling.
  - Sampling was coupled to a consecutive high pressure liquid chromatography (HPLC) separation of the extract components.
  - Detection was realized by electrospray ionization mass spectrometry (ESI/MS).
- **RESULTS:**
  - Four different organs (brain, lung, kidney and liver) from whole-body thin tissue sections of propranolol dosed and control mice were examined.
  - The parent drug was observed in the chromatograms of the surface sampling extracts from all the organs examined.
  - Two isomeric phase II metabolites of propranolol (an aliphatic and an aromatic hydroxypropranolol glucuronide) were observed in the chromatograms of the extracts from lung, kidney and liver.
  - These drug and metabolite data and results achieved by analyzing similar samples by conventional extraction of the tissues and subsequent HPLC/MS analysis were consistent.

INTRODUCTION

- Direct liquid extraction based surface sampling probes [1] have shown success in the analysis of both drugs and phase II metabolites from animal thin tissue sections. [2-4].
- The liquid extraction based surface sampling methods allow for the easy incorporation of a liquid based separation after the surface sampling process.
- In this work we report on
  - adapting a commercially available autosampler to perform direct liquid microjunction surface sampling
  - implementation of a separation step into this liquid extraction-based surface sampling process
  - separation and detection of two isomeric phase II metabolites of propranolol (an aliphatic and an aromatic hydroxypropranolol glucuronide) during direct surface sampling of lung, kidney and liver of a mouse thin tissue section.

EXPERIMENTS

CHEMICALS. HPLC grade acetonitrile (ACN) and water were purchased from Burdick & Jackson (Muskegon, MI, USA). Formic acid (FA) (≥ 96% purity) was purchased from Sigma-Aldrich (St. Louis, MO, USA). Propranolol hydrochloride (Acros Organics, Morris Plains, NJ, USA) was obtained commercially and used without further purification.



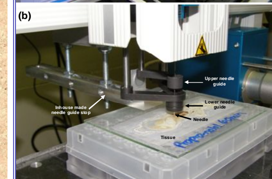
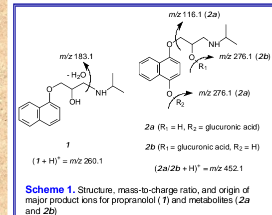


Figure 1. Photographs (a) of the surface sampling system utilizing an HTC-PAL autosampler coupled to HPLC/MS and (b) of the injection needle assembly of the HTC-PAL autosampler showing the syringe needle in surface sampling position above a thin tissue section on tape secured onto a glass slide/96-well plate for surface analysis.



Scheme 1. Structure, mass-to-charge ratio, and origin of major product ions for propranolol (*1*) and metabolites (*2a* and *2b*)

THIN TISSUE SECTION PREPARATION. Preparation and handling of mouse (male CD-1, Charles River Laboratories) whole-body thin tissue sections on tape (propranolol, cpd *1*, administered intravenously via the tail vein at 7.5 mg/kg as an aqueous solution in 0.9% NaCl and sacrificed at 60 min post-dose) have been described in detail elsewhere.[4]

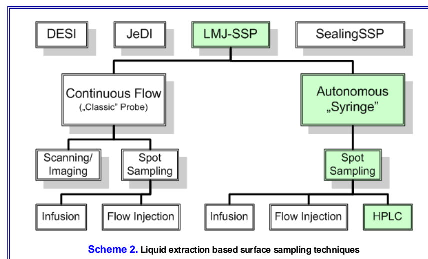
INSTRUMENTATION. A thin tissue section on tape was first secured with clear tape onto a 3" × 4", 1.2-mm thick glass slide, then the glass slide was secured with clear tape onto the top of a typical 96-well plate. The plate was then placed onto a plate holder which was secured onto an MS2000 x, y, z robotic platform (Applied Scientific Instrumentation Inc., Eugene, OR). An HTC PAL autosampler (LEAP Technologies Inc., Carrboro, NC) was used for liquid handling during surface sampling. The x, y, z stage and the autosampler were both secured onto the same 24" × 24" aluminum breadboard (Thorlabs, Newton, NJ). This was done to fix the position of the two x, y, z robots (i.e. the stage and the autosampler) to each other and to minimize shaking of the system during movement of the autosampler arm. Also, the vial holder of the autosampler was removed and replaced with an in-house made "needle guide stop" (**Figure 1**).

The autosampler was coupled to an Agilent 1200 HPLC system (Agilent Technologies, Santa Clara, CA) and to a 4000 QTRAP® mass spectrometer (AB SCIEX, Concord, Ontario, Canada). Compounds extracted from the surface using 90/10/0.1 (v/v/v) water/ACN/FA were injected onto a Synergi Hydro-RP HPLC column (50 x 2 mm, 4 µm particle size; Phenomenex, Torrance, CA). HPLC separation solvents A and B were water and ACN, respectively, both with 0.1% (v/v) FA. The 5-min-long gradient separation included the following steps: 0-0.5 min: constant 90/10 (v/v) A/B; 0.5-3 min: linear gradient from 90/10 (v/v) A/B to 35/65 (v/v) A/B; 3-3.5 min: linear gradient from 35/65 (v/v) A/B to 10/90 (v/v) A/B; 3.5-3.6 min: linear gradient from 10/90 (v/v) A/B to 90/10 (v/v) A/B; and 3.6-5 min: constant 90/10 (v/v) A/B. Solution flow rate was 200 µL/min.

Three selected reaction monitoring (SRM) transitions were monitored using positive ion mode ESI with an emitter voltage of 5 kV and turbo sprayer heater temperature of 300 °C. These SRM transitions included *m/z* 260.1 → 183.1 (collision energy (CE) = 27 eV; declustering potential (DP) = 60 V) for 1, and *m/z* 452.1 → 116.1 and *m/z* 452.1 → 276.1 (both CE = 35 eV and DP = 60 V) for the hydroxypropranolol glucuronides. **Scheme 1** shows the compound structures and the monitored precursor and product ions.

CONVENTIONAL HPLC-MS/MS ANALYSIS. Brain, kidney, liver, and lung were excised and rinsed in saline from a mouse. Organs were homogenized in three portions (w/v) water using a small tissue homogenizer. Samples were extracted in two portions 50/50 (v/v) ACN/water, dried down under N_2, reconstituted in 10 mM ammonium acetate and injected (100 mL) into an Agilent HPLC. The HPLC method was run using a Phenomenex® Luna C18 150 × 2 mm column (pore size: 100 Å, particle size: 5 µm), flow rate 0.5 mL/min, mobile phase A: 10 mM ammonium acetate, mobile phase B: acetonitrile, 7 min at 100% A, 7 to 45 min gradient to 85% B, and return to 100% A for 5 min. MS detection was accomplished using a Finnigan TSQ Quantum Ultra MS (positive ion mode ESI).

RESULTS AND DISCUSSIONS



Scheme 2. Liquid extraction based surface sampling techniques

OPERATION OF THE LIQUID MICROJUNCTION-HPLC/MS SURFACE SAMPLING SYSTEM. Customized robotic (syringe needle) movements and custom liquid handling for a surface analysis was setup in the HTC PAL autosampler instrument control software (LEAP Shell3): the autosampler was instructed to carry out the surface sampling process (**Figure 2**) and inject the sample onto the HPLC column.

Figure 2. Sequential steps of the surface sampling process showing (a) the syringe needle approaching the surface, (b) a liquid microjunction created between the needle and a liver thin tissue section (inset shows a magnified view) and (c) sample aspirated back into the needle and ready to be injected onto an HPLC column.

- Results of separate HPLC/MS analyses of homogenates of the four organs of interest confirmed the existence of these same two metabolites in lung, liver and kidney (**Figure 4**).
- Sampling the same organs of a control tissue section did not produce signal above background levels for either the parent drug or the metabolites (data not shown).
- Recently, glucuronidation of 4'-hydroxypropranolol was examined by Salomonsson et al.[6] They found two isomeric forms of 4'-hydroxypropranolol glucuronide with different retention times when 4'-hydroxypropranolol was incubated with uridine 5'-diphosphoglucuronic acid (UDPGA, glucuronidation agent) and uridine 5'-diphosphoglucuronyl transferase (UDPGT, microsomal glucuronidation enzyme).
- Using separation conditions similar to those used here, they identified the metabolite with a shorter retention time as an aromatic O-glucuronide (glucuronic acid attached via the aromatic OH group), and the one with longer retention time as an aliphatic O-glucuronide (glucuronic acid attached via the aliphatic OH group).
- We made the tentative assignments of the metabolites at retention time of 2.86 and 3.32 mins as compounds *2a* and *2b*, the aromatic and the aliphatic 4'-hydroxypropranolol glucuronides, respectively.
- Low signal levels prevented us from collecting quality product ion spectra of these two metabolites for direct comparison with the spectra published by Salomonsson et al.[6]
- However, we did note that the product ion at *m/z* 116 (detected by SRM transition *m/z* 452.1 → 116.1) was more abundant for metabolite *2a* than *2b* (as can be observed in the chromatograms shown in **Figure 5**).
- The product ion spectra of these two different glucuronides published by Salomonsson et al.[6] also showed this same difference supporting our tentative identification.

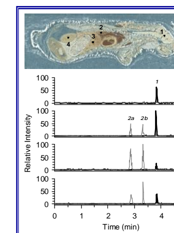


Figure 3. a.) Photograph of a propranolol dosed mouse (7.5 mg/kg, I.V. dosed, sacrificed 1 hour after dose) whole-body thin tissue section on tape showing sampled locations of (1) brain, (2) lung, (3) liver and (4) kidney tissue. The surface spots in (a) were analyzed using the autosampler/HPLC-MS surface sampling system. Signal levels for (black line) propranolol (cpd *1*, m/z 260 → 183) and (gray line, cpds *2a* and *2b*) hydroxypropranolol glucuronide (m/z 452 → 276) were recorded during 5 min HPLC/MS run of b.) brain, c.) lung, d.) liver and e.) kidney samples. Measured signal intensities were normalized individually for the drug and for the metabolite using the highest signal of a transition observed during the entire analysis of the four samples (3400 cps and 1600 cps, respectively).

- **THIN TISSUE SECTION ANALYSIS.** A whole-body thin tissue section from a mouse that had been administered propranolol was examined. The specific areas sampled are annotated in the photograph of the tissue section shown in **Figure 3a.**
- The chromatograms obtained monitoring the specific SRM transitions for propranolol (cpd *1*, *m/z* 260.1 → 183.1) and the potential hydroxypropranolol glucuronide metabolites (cpds *2a* and *2b*, *m/z* 452.1 → 276.1) from the surface sampling extracts of brain, lung, liver and kidney are shown in **Figure 3b-e**, respectively.
- Propranolol was observed in the chromatograms of the extracts from all four organs examined, with the highest levels recorded in the brain and lung.
- These results were in line with those we had observed previously using other liquid extraction based surface sampling methods to analyze tissues of mice dosed following the same protocol as described here.[3-5]
- Most importantly, the chromatograms revealed in all organs, except the brain, the presence of two different hydroxypropranolol glucuronide metabolites. These two glucuronides appeared at retention times of 2.86 and 3.32 mins, respectively.
- In our prior surface sampling work [3-5], we detected hydroxypropranolol glucuronide in these same tissues, but could not confirm if one or more particular isomers were present.

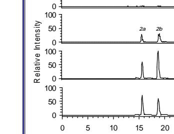


Figure 4. Signal levels for hydroxypropranolol glucuronide (m/z 452 → 276) recorded during HPLC/MS run of (a) brain, (b) lung, (c) liver and (d) kidney homogenates of a propranolol dosed mouse (7.5 mg/kg, I.V. dosed, sacrificed 1 hour after dose).

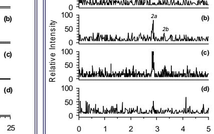


Figure 5. Signal levels for hydroxypropranolol glucuronide (*m/z* 452 → 116) were recorded during 5 min HPLC/MS run of (a) brain, (b) lung, (c) liver and (d) kidney samples (surface spots are shown in **Figure 3a**).

CONCLUSION

- **The use of an autosampler/HPLC-MS system for direct liquid microjunction surface sampling of thin tissue sections with subsequent separation and mass spectrometric analysis of the extract was demonstrated.**
- **The system was used to sample four major organs, viz., brain, lung, kidney and liver of whole-body mouse thin tissue sections.**
  - **Propranolol was detected in all organs examined.**
  - **Two isomeric hydroxypropranolol glucuronide metabolites, at different retention times, were detected in lung, kidney and liver of the same tissue.**
  - **The metabolite with shorter retention time was tentatively identified as an aromatic O-glucuronide and the one with longer retention time as an aliphatic O-glucuronide.**
- **FUTURE WORK.**
  - **Investigation of alternative chromatographic separation phases for the present application (HILIC column using what we know to be a more optimum extraction solvent for the drug and metabolites of interest, i.e. 20/80/0.1 (v/v/v) water/ACN/FA).**
  - **Other types of chromatography, for example, those aimed at separation of the enantiomers of chiral drugs and their metabolites will be exploited in direct surface sampling HPLC-MS analysis of thin tissue sections.**
  - **In general, direct and efficient sampling from thin tissue sections by liquid extraction followed by liquid phase separation for drug discovery purposes.**

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