**Quantitation of Repaglinide and Metabolites in Mouse Whole-Body
Thin Tissue Sections using Droplet-Based Liquid Microjunction
Surface Sampling-High-Performance Liquid Chromatography-
Electrospray Ionization Tandem Mass Spectrometry**

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Abstract

Herein, quantitation aspects of a fully automated autosampler/HPLC-MS/MS system applied for unattended droplet-based surface sampling of repaglinide dosed thin tissue sections with subsequent HPLC separation and mass spectrometric analysis of parent drug and various drug metabolites was studied. Major organs (brain, lung, liver, kidney, muscle) from whole-body thin tissue sections and corresponding organ homogenates prepared from repaglinide dosed mice were sampled by surface sampling and by bulk extraction, respectively, and analyzed by HPLC-MS/MS. A semi-quantitative agreement between data obtained by surface sampling and that by employing organ homogenate extraction was observed. Drug concentrations obtained by the two methods followed the same patterns for post-dose time points (0.25, 0.5, 1 and 2 h). Drug amounts determined in the specific tissues was typically higher when analyzing extracts from the organ homogenates. In addition, relative comparison of the levels of individual metabolites between the two analytical methods also revealed good semi-quantitative agreement.

Keywords

liquid microjunction, droplet-based liquid extraction, quantitation, autosampler, spatial distribution, repaglinide.

1. Introduction

Tissue distribution studies in animals are an indispensable part of the drug discovery and development pipeline [1]. The information these studies provide is important for the understanding and prediction of pharmacological target engagement, toxicological target tissue exposure, and drug disposition.

A number of different technologies are currently used to measure drug and metabolite distributions directly from tissues. For example, quantitative whole-body autoradiography (QWBA) [2,3] and matrix-assisted laser desorption/ionization mass spectrometry (MALDI-MS) [4,5,6] are used to analyze drug dosed thin tissue sections. High performance liquid chromatography-mass spectrometry (HPLC-MS) is used to measure these species in whole organ tissue homogenates [1] or in punched thin tissue sections [7]. However, each of these approaches has analytical shortcomings. QWBA requires the use of an often expensive radiolabeled drug and it is unable to distinguish between parent drug and its metabolites. MALDI-MS analysis requires coating the sample with a chemical matrix prior to MS analysis and it is not well-suited to detect fragile molecules like phase II metabolites [5]. In addition, MALDI lacks the capability to transfer the ablated material for post-sampling processing such as an HPLC separation. Post-sampling processing is advantageous in dealing with complex sample matrices, multiple analytes, and/or for separation and identification of isomeric compounds not distinguishable by MS methods alone. Such is the case when employing HPLC-MS analysis with the above mentioned punched samples or whole organ tissue homogenates. The use of HPLC-MS also enables absolute analyte quantitation. However, HPLC-MS analysis of punched samples and tissue homogenates typically

requires extensive sample handling, extraction, and cleanup steps. While an unattended workflow might be employed for this multi-step process using conventional laboratory preparative schemes and robots, simplifying and speeding up the analytical workflow is desirable.

Liquid extraction based surface sampling probes [8] have been increasingly applied with tandem MS (MS/MS) for sampling and detecting drugs [9] and phase I,[10,11,12] and phase II metabolites [13,14,15,16,17,18,19,20] directly from animal thin tissue sections. The rise in interest in this spatially resolved surface sampling/analysis methodology is due, in part, to the enhanced sensitivity compared to other ambient surface sampling techniques [21,22], and to the integration with HPLC-MS/MS systems [16,17,18,19,20,23,24]. Comparative relative quantitation of drug and metabolites distributions determined by the liquid extraction based surface sampling probes, by QWBA and HPLC-MS [17], and QWBA and radio thin-layer chromatography [18] analyses have shown good agreement.

Repaglinide is an insulin secretagogue used in the treatment of type 2 diabetes. In humans, repaglinide is rapidly absorbed and cleared with a time to maximum serum concentration of 0.5 hours (oral solution) and a terminal half life of 0.53 hours. A dicarboxylic acid metabolite, M2, is a major metabolite in humans. Other metabolites include hydroxylated metabolite M4 and an acyl glucuronide metabolite M7. It is of our interest to study the tissue distribution of repaglinide in a time scale that is consistent with its rapid absorption and elimination [25,26]. In this paper, we quantitate repaglinide and its metabolites in organs of whole-body mice thin tissue sections using a fully automated droplet-based liquid microjunction surface sampling probe (LMJSSP)-HPLC-

MS/MS system [16,17,18,19,20,27] and compare those results with quantitation using HPLC-MS/MS analysis of bulk extracts from organ homogenates. Absolute drug concentrations obtained by the two methods differed but showed good relative agreement with concentrations determined generally higher with the organ homogenate extracts. Reasons for differences observed in concentration values and strategies to correct for them are discussed in detail.

2. Experimental

2.1. Reagents and materials

Repaglinide (cpd **1**) and Cremophor[®] RH 40 were purchased from Sigma Aldrich (St. Louis, MO, USA). Repaglinide-d5 (cpd **1a**) was purchased from Toronto Research Chemicals (North York, Ontario, Canada). Blank BALB/c mouse plasma was purchased from BioreclamationIVT (Westbury, NY, USA). All organic solvents and water were of HPLC grade or better and purchased from Sigma Aldrich.

2.2. Sample preparation

2.2.1. Mouse dosing

All animals used in this study were handled in accordance with the Guide for the Care and Use of Laboratory Animals [28]. Before the study initiation, the study protocol was approved by the Institutional Animal Care and Use Committee. Male BALB/c mice (n = 4, 18-25 g body weight) were purchased from Hilltop Laboratories (Scottsdale, PA, USA). Animals were fasted overnight (at least 12 h) prior to, and throughout the study following dose administration. Each mouse received a single oral dose of repaglinide at

1 mg/kg as a solution in 25/75 (w/w) Cremophor[®] RH 40/water. One mouse per time point was euthanatized by deeply anesthetizing at 0.25, 0.5, 1 and 2 h post-dose. All carcasses were frozen at -70 °C dry ice/ethanol bath and stored at -80 °C until required for embedding.

2.2.2. Preparation of whole-body thin tissue sections for surface sampling analysis

An individual mouse carcass in a sagittal position was embedded in a chilled 2% carboxymethylcellulose matrix and mounted on a microtome stage maintained in a dry ice/ethanol bath at -70 °C. Whole-body sagittal tissue sections of 50 µm thickness were collected on 845 Scotch[®] tape (3M, St. Paul, MN) using a cryomicrotome (model Leica CM3600, Leica Microsystems Inc., Bannockburn, IL) at -20 °C. Sections at various levels were collected to include the major organs investigated. The sections were dried in the cryomicrotome at -20 °C for at least 48 h before analysis.

2.2.3. Preparation of liver homogenate thin sections for surface sampling calibration curve

Liver was homogenized in water (1-to-1 ratio (w/w)) using an Omni TH homogenizer (OMNI International, Waterbury CT, USA) with final repaglinide concentration ranging from 50 nM to 5 µM in the homogenate. (Liver tissue was used in the preparation of calibration standards since its density was similar to the majority of tissues in the whole-body section.) Holes 2.5 mm in diameter were drilled in a frozen tofu block (ca. 100 x 50 x 12.5 mm in size, purchased locally) then the liver homogenate

standards were added into the holes while the tofu block was on dry ice. For this reason, no significant loss of drug to the tofu block was expected during preparation of the tissue section standards. After the liver homogenates completely froze, the tofu block was embedded in 2% carboxymethylcellulose matrix at -20 °C and sections of 50 µm thickness were collected on tape and dried in the cryomicrotome at -20 °C for at least 48 h before the surface sampling analysis. These samples were used to quantify repaglinide in mouse whole-body thin tissue section samples measured by the surface sampling system.

2.2.4. Preparation of samples from bulk extraction of organ homogenates

Major organs (brain, lung, liver, kidney, muscle) were excised from each frozen carcass after preparation of whole-body tissue sections and were stored at -20 °C until required for homogenization. Each organ was homogenized in water with 1-to-1 ratio (w/w). Organ homogenates 50 µL in volume were mixed with 50 µL of mouse blank plasma and 200 µL of acetonitrile (ACN) containing 20 nM of repaglinide-d5 internal standard (IS, cpd **1a**). The resulting samples were mixed well and shaken for 2 min with a mechanical shaker extensively, then centrifuged for 10 min at 4000 rpm. 240 µL of supernatants were mixed with 80 µL of water and transferred into a 96-well plate.

2.2.5. Preparation of samples for bulk extraction calibration curve

100 µL of mouse blank plasma samples with repaglinide concentrations ranging from 0.3 nM to 10 µM were mixed with 200 µL aliquots of ACN containing 20 nM of IS. The samples were mixed well, shaken for 2 min with a mechanical shaker extensively,

then centrifuged for 10 min at 4000 rpm. 240 μ L of supernatants were mixed with 80 μ L of water and transferred into a 96-well plate. These samples were used to quantify repaglinide in bulk extraction analysis of organ homogenate samples.

2.3. Sample analysis

2.3.1. Whole-body thin tissue section analysis by droplet-LMJSSP-HPLC-MS/MS

Whole-body and tofu-embedded liver homogenate thin sections were analyzed using droplet-based LMJSSP-HPLC-MS/MS. Briefly, the tissue section was attached to a 3 x 4 in. glass slide using double sided tape, and then the glass slide was mounted in a microtiter plate size “universal” sample holder [15] (Advion BioSciences, Inc., Ithaca, NY, USA). The optical image of the sample was acquired using a scanner (ScanMaker 3630, Microtek Lab, Inc., Santa Fe Springs, CA, USA) with software developed at ORNL (LMJ Points Plus[®] V2.98). After optical image acquisition, the sample section on the sample holder was placed into a tray of a CTC PAL autosampler (LEAP Technologies Inc., Carrboro, NC, USA) and the analysis locations in sample were selected using the same software. An extraction solvent containing 1/1 (v/v) ACN/water containing 100 nM IS of cpd **1a** was used for surface extraction. The extraction solvent vial was placed in a vial holder which was located in the CTC PAL autosampler. The surface sampling process was similar to that described previously [16,17,18,27]. Briefly, the autosampler first drew 5 μ L of solvent from the extraction solvent vial followed by switching the syringe needle into surface sampling mode using *Wash1*, location of the first washing solution. As illustrated in Scheme 2, then the needle moved to the section and 5 μ L extraction solvent was dispensed onto a previously selected

surface spot creating a liquid junction of approximately 2 mm in diameter and 100–300 μm in thickness (needle-to-surface distance). After 5 second extraction time, the liquid was aspirated back into the syringe and the full 5 μL samples was injected onto an Atlantis[®] C18 HPLC column (50 mm x 2.1 mm, 3 μm ; Waters, Milford, MA, USA) for subsequent HPLC/MS analysis employing a Shimadzu HPLC system equipped with two LC-10AD VP pumps (Shimadzu Scientific Instruments, Inc., Kyoto, Japan) to deliver the separation solvents coupled to an 4000 QTRAP hybrid triple quadrupole/linear ion trap mass spectrometer (AB SCIEX, Concord, Ontario, Canada). HPLC separation solvents A and B were 100/0.1 (v/v) water/FA and 100/0.1 (v/v) ACN/FA, respectively. The 5-min-long gradient separation starting from 70% A included the following steps: 0-2.8 min: linear gradient to 10% A; 2.8-3.3 min: 10% A; 3.3-3.4 min: linear gradient to 70% A; 3.4-5 min: 70% A. Solution flow rate was 300 $\mu\text{L}/\text{min}$.

Selected reaction monitoring (SRM) transitions of m/z 453.3 $\rightarrow$ 230.3 (repaglinide, cpd **1**), m/z 458.3 $\rightarrow$ 230.3 (repaglinide-d5, cpd **1a**), m/z 629.3 $\rightarrow$ 435.3 (metabolite **M7**), m/z 469.3 $\rightarrow$ 246.3 (metabolite **M4**) and m/z 485.3 $\rightarrow$ 262.3 (metabolite **M2**) were monitored using positive ion mode ESI with an emitter voltage of 5 kV and employing a collision energy (CE) of 35 eV and a declustering potential (DP) of 80 V. Dwell time was 55 ms for each transition monitored. Turbo sprayer heater temperature was 300 $^{\circ}\text{C}$. Scheme 1 shows the compound structures, mass-to-charge ratio and the origin of the major product ions monitored in the tandem mass spectrometry measurements.

2.3.2. HPLC-MS/MS analysis of samples from bulk extraction of organ homogenates

Samples from bulk extraction of organ homogenates (5 μ L of each) were analyzed using HPLC separation and MS/MS detection conditions identical to that of mouse whole-body thin section analysis.

3. Results and discussion

3.1. Absolute quantitation of repaglinide in organs from thin tissue sections and organ homogenates

Figures 1a and b show signal levels recorded during a 5 min HPLC-MS/MS analysis of a liver sample (0.5 h post dose) for parent drug repaglinide and internal standard repaglinide-d5, respectively, using droplet-based surface sampling methodology. Figures 1f and g show signal levels when a homogenate of the same liver was analyzed by bulk extraction HPLC-MS/MS. Gray sections represent the retention time (R_t) ranges used for peak integration (R_t =1.6-2 min for both **1** and **1a**). Using droplet-LMJSSP-HPLC-MS/MS liver, kidney, muscle, brain and lung at each post-dose time points (i.e. 0.25, 0.5, 1 and 2 h) were analyzed in triplicate (three thin tissue sections, one sample location for a given tissue type in each section) and calculated drug content is shown in Figure 2a. The calculation employed a calibration curve obtained from analysis of thin sections prepared from liver homogenates with 50 nM - 5 μ M repaglinide (see Supplemental Figure S1). Using bulk extraction HPLC-MS/MS liver, kidney, muscle, brain and lung homogenates were analyzed in duplicates (two separate extractions each from a given tissue homogenate) at each post-dose time

points (i.e. 0.25, 0.5, 1 and 2 h) and calculated drug content is shown in Figure 2b. The calculation employed a calibration curve obtained from analysis of mouse plasma as matrix and concentrations of repaglinide ranged from 0.3 nM to 10 μ M (see Supplemental Figure S2). Good relative agreement between data obtained by the two methods was obvious. To better aid quantitative comparison of calculated drug content obtained using surface sampling and bulk extraction of tissue homogenates, ratios of corresponding values were calculated and are plotted in Figure 2c. These values were between 0.1 and 2.1, and in general they indicated that calculated drug content was lower (ratio<1) when using surface sampling than that using bulk extraction; only two time points (0.5 and 2h) for muscle and one time point for lung (1 h) exhibited the opposite trend. Another observation was that ratio values averaged using all time points for the organs (indicated by the wide empty bars with corresponding error bars in Figure 2c) was around 0.4 for the more dense tissues (kidney, liver and brain) and around 1 for the less structured ones (muscle and lung). Good reproducibility among biological replicates (%RSD<30%) suggested that the source of these observed concentration differences must have been biological in nature and/or related to sample preparation and not due to technical/experimental error.

Based on these observations, there are several possible sources of the quantitative disagreement between the employed methods. First, the calibration curve used with the surface sampling method was obtained with tissue homogenates and applied for un-homogenized tissue sections. Most likely, extraction efficiency from homogenized thin tissue sections used for calibration curve creation were higher than that from non-homogenized whole-body ones using the current extraction conditions. In

addition, because correction factors were applied based on the responses from different tissue homogenates, there could be differential biases in quantitation if extraction efficiencies are different between each tissue homogenates and real tissue. This bias seems to be consistent with some tissues (e.g. liver, kidney and brain) but different in other tissues such as muscle and lung. Interestingly, muscle and lung are both highly fibrous but contain less lipids [29] than the other tissues investigated. It may be possible that difference in fiber and/or lipid content could be a source of such differential bias in tissue extraction although further experiments are needed to confirm such hypothesis. Nevertheless, applying the given calibration curve for whole-body tissue sections likely resulted in a tissue dependent underestimation of drug content in the latter leading to the generally observed lower values with the surface sampling method. Another source of potential bias is from the relative water content of thin slices between tofu slices and whole body slices. Although all slices were processed in the same fashion and dried before analysis, the relative water content was not compared. Therefore, different water contents were possible between slices and even between different tissue types on the same slice, resulting in differences in nominal concentrations in standard curves for tofu blocks therefore biases in quantitative results. Determination of water content in future experiments might correct for this potential bias in concentrations. Furthermore, bulk extraction of organ homogenates provided the average concentration in the amount of tissue used for sample preparation (1 g, that is about 1 mL of tissue in the current study), while surface sampling analyzed an area of about 2 mm in diameter, that is about 0.16 μ L in volume employing 50- μ m-thick tissues.

In other words, true spatial distribution differences in the concentrations of the drug in the tissues are reflected in the data from the surface sampling based method.

3.3. Relative quantitation of repaglinide metabolites in organs from thin tissue sections and organ homogenates

In contrast to the analysis of the parent drug, calibration curves were not created for repaglinide metabolites due to lack of standards. Thus, only relative quantitation of these compounds was attempted. Figures 1c, d and e show signal levels recorded during a 5 min HPLC-MS/MS analysis of a liver sample (0.5 h post dose) for repaglinide metabolites **M7**, **M4** and **M2**, respectively, using thin tissue sections with surface sampling. Corresponding signal levels for the same metabolites when homogenate of the same liver was analyzed by bulk extraction HPLC-MS/MS are shown in Figures 1h, i and j, respectively. Gray sections indicate R_t ranges used for peak integration ($R_t=1.2$ -1.6 min for **M7**, $R_t=1.35$ -1.75 min for **M4** and $R_t=1.85$ -2.25 min for **M2**). The integrated peak areas of all three metabolites were converted into arbitrary concentration values (c') shown in Figure 3 using the corresponding calibration curves obtained for the parent drug above. For these reasons, the obtained c' concentration values should only be used for relative comparison of the levels of individual metabolites between the two analytical methods. This comparison shows good relative agreement between data obtained by the two methods similar to the results obtained for the parent drug. Higher levels of metabolites were determined using organ homogenates most likely due to reasons discussed above for the parent drug.

4. Conclusions

In this paper, we described quantitation aspects of a fully automated droplet-based HPLC-MS/MS system applied for unattended surface sampling of repaglinide dosed thin tissue sections with subsequent HPLC separation and tandem mass spectrometric analysis of parent drug and its metabolites. Good relative agreement for tissue concentrations of the drug and metabolites was obtained with the two methods. In almost all cases, however, the specific tissue concentrations determined were higher when using organ homogenates compared to selected spot sampling of regions within the thin tissue sections of the same organ. This observation was attributed to the calibration curve used with the surface sampling method being obtained with tissue homogenates and applied for un-homogenized tissue sections. Most likely the extraction efficiency from a tissue section was lower than that of a homogenized tissue thus the calibration curve resulted in the underestimation of drug content and consequently in the observed lower values with the surface sampling method. In addition, spatial distribution of repaglinide (and metabolites) presumably affected their concentrations obtained by the two methods due to different volumes sampled by them.

Based on the results of this work, future studies on absolute quantitation using the droplet-based surface sampling system will focus on extraction strategies to achieve 100% extraction efficiency (i.e., recovery) of targeted analytes from thin tissue sections. This path will involve comparative quantitation of thin sections by direct surface sampling and by bulk extraction of tissue punches only a few mm in diameter of adjacent thin sections. This methodology would allow the evaluation of extraction efficiencies without spatial distribution of the analyte of interest affecting the results.

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1

$$(M + H)^+ = m/z \ 453.3$$

1a

$$(M + H)^+ = m/z \ 458.3$$

M7

$$(M + H)^+ = m/z \ 629.3$$

M4

$$(M + H)^+ = m/z \ 469.3$$

M2

$$(M + H)^+ = m/z \ 485.3$$

Scheme 1. Structure, mass-to-charge ratio, and origin of major product ions for repaglinide (**1**), repaglinide-d5 (**1a**), repaglinide acyl-glucuronide (**M7**), 3'-hydroxyl repaglinide (cpd **M4**) and 2-despiperidyl-2-(5-carboxypentylamine repaglinide) (**M2**).

Scheme 2. Schematic of the surface sampling process including (a) the positioning of the syringe needle 100-300 μm above a defined surface spot, (b) dispensing a discrete volume of extraction solvent onto a surface creating a liquid junction between the needle and the surface, (c) dissolution of the analyte in the extraction solvent, (d) the liquid is drawn back into the syringe needle after a predefined extraction time, (e) the liquid is loaded into the sample loop and (f) injected for a consecutive HPLC-MS analysis.

Figure 1.

Figure 2.

Figure 3.

FIGURE CAPTIONS

Figure 1. Signal levels recorded during a 5 min HPLC-MS/MS analysis for (a,f) parent drug repaglinide (cpd **1**), (b,g) internal standard repaglinide-d5 (cpd **1a**), and metabolites (c,h) repaglinide acyl-glucuronide (cpd **M7**), (d,i) 3'-hydroxyl repaglinide (cpd **M4**) and (e,j) 2-despiperidyl-2-(5-carboxypentylamine repaglinide) (cpd **M2**) obtained using thin tissue sections with surface sampling and traditional tissue homogenates extraction, respectively. Gray sections show the time ranges used for peak integration: $R_t=1.6-2$ min for **1** and **1a**, $R_t=1.2-1.6$ min for **M7**, $R_t=1.35-1.75$ min for **M4** and $R_t=1.85-2.25$ min for **M2**.

Figure 2. Averaged tissue concentration of repaglinide (cpd **1**) obtained using (a) thin tissue sections and surface sampling and (b) traditional tissue homogenates extraction at (filled bars) 0.25 h, (empty bars) 0.5 h, (hatched bars) 1 h and (cross hatched bars) 2h after dose in various organs. (c) Ratios of concentrations shown in (a) versus those presented in (b), with average ratios and standard deviations indicated by wide empty bars and error bars for the individual organs.

Figure 3. Averaged relative concentration of metabolites **M7**, **M4** and **M2** in liver obtained using (a) thin tissue sections and surface sampling and (b) traditional tissue homogenates extraction at (filled bars) 0.25 h, (empty bars) 0.5 h, (hatched bars) 1 h and (cross hatched bars) 2h after dose.