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Application of exogenous mixture of glutathione and stable isotope labeled glutathione for trapping reactive metabolites in cryopreserved human hepatocytes. Detection of the glutathione conjugates using high resolution accurate mass spectrometry

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ABSTRACT

Metabolites (including reactive metabolites) of troglitazone were generated by incubation with cryopreserved human hepatocytes and trapped in the presence of an exogenous mixture of unlabeled and stable isotope labeled (SIL: [1,2-^{13}C, ^{15}N]-glycine) glutathione (GSH/SIL-GSH). The incubation samples were analyzed using liquid chromatography–high resolution accurate mass spectrometry (LC–HRAMS) implemented on a LTQ Orbitrap mass spectrometer. The GSH conjugates of the reactive metabolites were detected via a characteristic mono-isotopic pattern (peaks separated by 3.0037 u). Analysis of the incubation samples led to detection of a number of previously described GSH conjugates, as well as two novel methylated GSH conjugates, which were partially characterized based on accurate mass measurements and MS/MS data. The addition of exogenous GSH led to an increase in the apparent level of detected GSH conjugates. Kinetic isotopic measurements showed that the rates of incorporation of exogenous GSH are conjugate-specific. In conclusion, this approach, based on the use of a mixture of GSH/SIL-GSH, allows facile capture and detection of reactive metabolites in human hepatocytes. Moreover, the data suggest that routine addition of glutathione to the assay medium may be advisable for experiments with cryopreserved hepatocytes.

1. Introduction

Metabolic activation of drugs, leading to generation of reactive metabolites followed by covalent binding to cellular macromolecules, could potentially be associated with idiosyncratic toxicity [1,2]. Since the formation of reactive metabolites is considered to be an unfavorable property of drug candidates, screening for reactive metabolites has become an integral part of the modern drug discovery process [3,4]. For the purpose of screening, reactive metabolites are typically generated in subcellular hepatic fractions (microsomes or S9) supplemented with NADPH and glutathione (GSH), trapped as GSH conjugates (due to their transient nature), and detected using various LC–MS methodologies [5,6]. Historically, LC–MS/MS methods developed for detection of GSH conjugates were based on MS/MS fragmentation pathways somewhat specific for the GSH moiety, the most prominent being neutral loss (NL) of 129 Da (NL = 129) in positive electrospray ionization mode [4] or precursor ion (Pr) scans of −272 and −254 Th (Pr = −272/−254) for negative ionization mode [6,7].

In order to increase selectivity in MS/MS detection, application of a mixture of unlabeled GSH and stable isotope labeled (SIL) GSH (SIL-GSH) has been reported [8–10]. The presence of characteristic signals in both channels "0" and "+3 u" (e.g., NL = 129 and NL = 132) helps rule out false positives.

The inherent disadvantage of MS/MS methodologies is their dependence on the MS/MS fragmentation pathway of the GSH moiety, which is often conjugate-specific. To overcome this limitation, an approach for detection of GSH conjugates via direct observation of the characteristic mono-isotopic pattern, arising from application of the mixture of GSH and SIL-GSH, has been reported. The survey MS detection could be implemented on a linear trap instrument [11–13] at unit resolution or, preferably, using high resolution accurate mass spectrometry (HRAMS) on an Orbitrap instrument to achieve superior selectivity [14].

To the best of our knowledge, all previous work describing the application of the SIL approach for detection of GSH conjugates was performed using subcellular fractions (liver microsomes or S9). Performing drug metabolism studies in intact hepatocytes could

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be advantageous since it is a more physiologically relevant model. Here we report the results of application of the SIL approach for detection of GSH conjugates in intact, cryopreserved human hepatocytes.

2. Materials and methods

2.1. Chemicals

All solvents (from VWR) were HPLC grade. The troglitazone was purchased from Toronto Research Chemicals, Inc. The unlabeled GSH (99% pure) was from Sigma, the SIL-GSH ([1,2-^{13}C, ^{15}N]-glycine), isotopic purity ~90%, was from Cambridge Isotope Laboratories.

2.2. Incubation with hepatocytes and sample preparation

Human cryopreserved hepatocytes (mixed gender, pool of three donors) were obtained from XenoTech (lot numbers H684, H789, and H886 in Pool I; HC3-3A, HC1-8, and HC5-14 in Pool II. The hepatocytes were thawed and prepared according to the vendor's

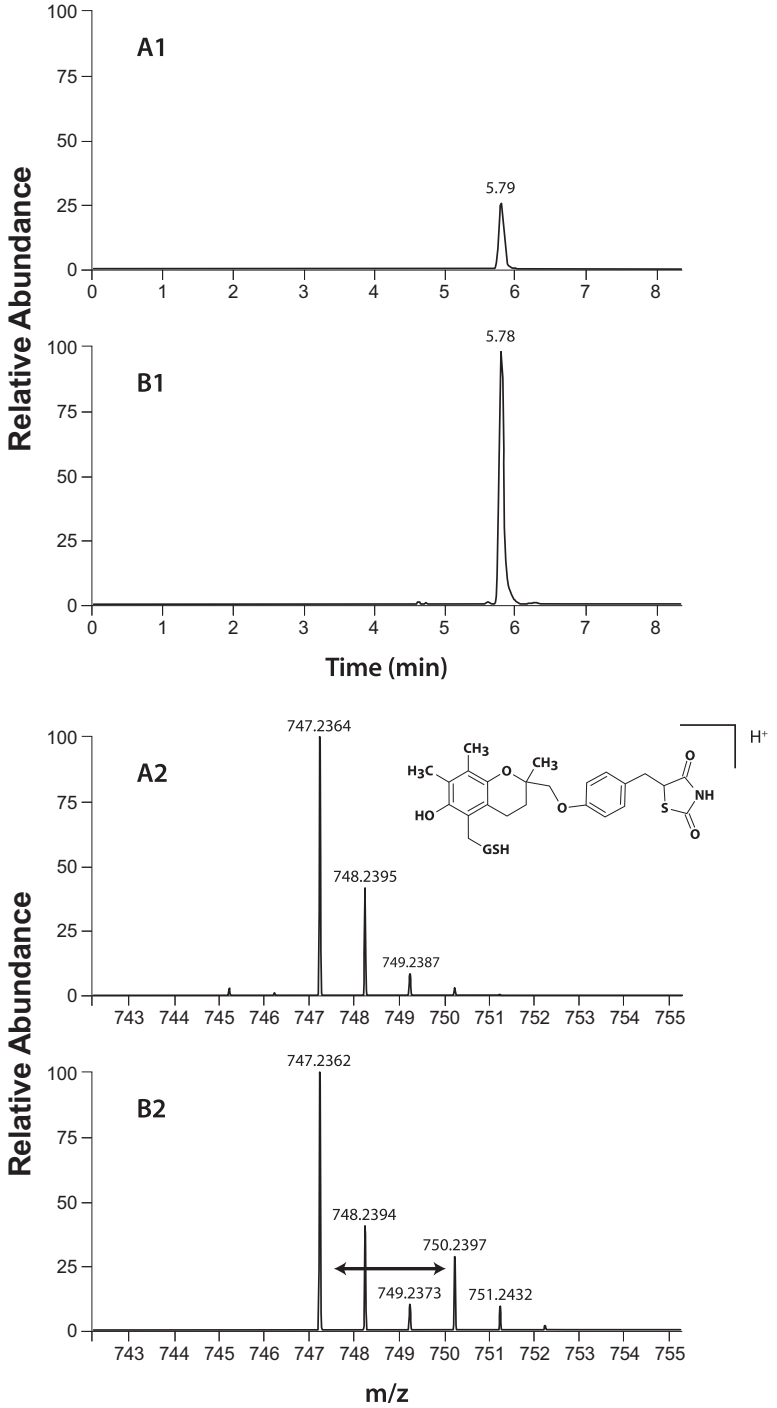

Fig. 1. XICs of known TGZ–GSH conjugate M5 at m/z = 747.2364, generated without (A1) and with (B1) the addition of GSH/SIL-GSH mixture. *Note*: all peaks in all figures were constructed using the sum of GSH- and SIL-GSH-derived molecular ions and are shown using the same scale for comparative purposes. HRAMS survey spectra of peaks of M5 generated without (A2) and with (B2) the addition of GSH/SIL-GSH mixture. Arrow indicates the characteristic mono-isotopic split (+3.0037). Inset: structure of M5 [15].