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Application of high-performance liquid chromatography–electrospray ionization–mass spectrometry to measure microsomal membrane transport of glucuronides

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Abstract

A primary reason for poor characterization of microsomal transport to date is the limitations of the measurement techniques used. Radiodetection provides sufficient sensitivity, but it can be applied only when labeled analogue is available. In this article, we report the novel application of high-performance liquid chromatography and electrospray tandem mass spectrometry (LC–MS/MS) in "rapid filtration" transport assays. The method was developed using glucuronides, but it is adaptable to any compound that can be measured with LC–MS/MS. Because of the high sensitivity and accuracy of this detection technique, the substrates can be used at their physiological concentration in the experiments. The new methodology does not require radiolabeling, so it remarkably widens the range of possible substrates to investigate and allows simultaneous detection as well as monitoring of substrate stability during the experiments.

Keywords: High-performance liquid chromatography; Electrospray ionization mass spectrometry; Transport; Membrane; Microsome; Rapid filtration; Glucuronide

Transport processes across the endoplasmic reticulum (ER)[1] membrane are key determinants of the luminal environment of the organelle, and they play an important role in the activity and specificity of certain luminal enzymes. The most widely applied in vitro ER transport assay is the "rapid filtration" measurement, which uses isolated ER-derived vesicles (microsomes) that maintain their original orientation (i.e., their intravesicular surface corresponds to the intraluminal side of the ER membrane). Rapid filtration is based on the possibility of retaining microsomes on filter membranes having sufficiently small pore size (usually <0.5 μm), allowing the quick separation of vesicle-associated molecules from the medium. After the microsomes have been filtered and quickly washed, the molecules still on the filter must be inside the microsomes or bound to their surface. The amount of intravesicular molecules can be calculated after the measurement is repeated using permeabilized microsomes, which hold only the membrane-bound compounds. The pore-forming alamethicin is often used as a membrane-permeabilizing agent in these experiments

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[1] *Abbreviations used:* ER, endoplasmic reticulum; MS, mass spectrometry; HPLC, high-performance liquid chromatography; MS/MS, tandem mass spectrometry; saccharolactone, D-saccharic acid 1,4-lactone; MOPS, 4-morpholinepropanesulfonic acid; APCI, atmospheric pressure chemical ionization; ESI, electrospray ionization.

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because it does not disintegrate the vesicles; hence, they are still suitable for filtration. The progress of uptake can be detected by adding the substance to the microsomes and processing samples after different periods of incubation. For release measurements, the microsomes are preloaded at a small volume, and the outward gradient is created by diluting the microsomes (at least 10-fold) at the beginning of the assay.

The application of physiological concentrations in rapid filtration assay is usually desirable; however, it often raises technical difficulties. Because the physiological concentrations of the studied molecules are typically low (in the micromolar range) and the average volume of vesicles is usually small (a few microliters per milligram protein), an extremely sensitive detection is needed for the measurements. Therefore, transport experiments of this kind to date have been limited by the availability of radiolabeled analogues. This limitation is especially apparent in the case of glucuronides. Only the transport of estradiol 17-glucuronide across the ER membrane has been characterized [1] despite the fact that a huge variety of glucuronides are synthesized in the lumen and thought to be transported to the cytosol [2].

Electrospray mass spectrometry (MS) often provides a good tool to detect trace amounts of molecules, including glucuronides [3,4]. The limit of detection is typically in the range of femtomoles, which is comparable to or better than that of radiodetection. Combined with high-performance liquid chromatography (HPLC), MS is suitable for detection and measurement of the investigated compounds from complex biological matrices. Although LC–MS is now widely used in chemistry, toxicology, and pharmacology, this approach has not been applied to microsomal transport assays.

In this article, we report the development of a novel method that is suitable for transport measurements (at physiological substrate concentrations) without using radiolabeled analogues. This method combines LC–tandem mass spectrometry (MS/MS) detection with rapid filtration assay for the measurement of transport both into and out of microsomes (ER-derived vesicles). The method was developed using glucuronides; however, it is not limited to these compounds and is adaptable for a wide range of molecules that can be quantitated with LC–MS. In addition to the significantly expanded substrate range for these experiments, this technique allows simultaneous transport measurement with several compounds such as concurrent measurement of efflux and influx, conduction of competitive inhibition experiments with measurement of both substrate and inhibitor, and monitoring of the stability and integrity of the substrates during the experiments. The use of this technique will greatly extend the ability to study microsomal transport.

Materials and methods

Materials

17-β-Estradiol, 17-(β-D-glucuronide), morphine, morphine 3-(β-D-glucuronide), phenolphthalein, phenolphthalein β-D-glucuronide, 4-nitrophenol, 4-nitrophenyl β-D-glucuronide, 1-naphthol, 1-naphthyl β-D-glucuronide, D-saccharic acid 1,4-lactone (saccharolactone), and 4-morpholinepropanesulfonic acid (MOPS) were purchased from Sigma–Aldrich (Gillingham, Dorset, UK). Acetonitrile (HPLC grade), formic acid, and all other chemicals (highest grade available) were purchased from Merck Eurolab (Poole, Dorset, UK).

Compound extraction

The studied compounds were extracted from mixed cellulose ester filter membranes (pore size 0.45 μM, 24 mm diameter, MF, Millipore) prepared in rapid filtration assay. Each filter was placed in 500 μl of extracting solution (e.g., 50% acetonitrile), and the solution was immediately vortexed for 10 s. It was allowed to stand for 30 min at room temperature to aid compound diffusion and then was vortexed for 10 s again. The sample was then frozen and kept at −20 °C until analysis. The sample was thawed, vortexed, and centrifuged at 14,000*g* for 6 min to pellet the partially disintegrated membrane. Then 200 μl of the solution was transferred to an HPLC vial for analysis.

Extraction efficiency experiments

To optimize extraction, the recoveries of substrates from the filters were compared using various extracting solutions. Each glucuronide was dissolved in MOPS–KCl buffer at 50 μM concentration with or without rat liver microsomes (1 mg protein/ml). Then 10 μl of these solutions was added directly to 500 μl of extracting solution (control) or onto MF filters. Glucuronides were then extracted from the filters with 500 μl of the given extracting solution as described above. Control and extracted samples were analyzed by LC–MS, and extraction efficiency was calculated as the percentage of glucuronides measured in extracted samples compared with controls.

LC conditions

LC conditions were optimized for each glucuronide from a standard program. The standard LC conditions consisted of the following: solvent A, 0.5% formic acid; solvent B, 0.5% formic acid in acetonitrile. The initial program consisted of a 1-min equilibration at 100% solvent A, followed by a 3-min gradient from 0 to 100% solvent B, and 1 min at 100% solvent B, followed by