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Short communication

Evaluation of evaporative light-scattering detection for metabolite quantification without authentic analytical standards or radiolabel☆

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Abstract

Development of a sensitive and specific technique for the quantitation of drug metabolites without the use of synthetic analytical standards or radiolabel would represent a major advance in preliminary route of metabolism screening in drug discovery. In this study, the ability of evaporative light-scattering detection (ELSD) to quantify metabolites of 7-ethoxycoumarin (EC) was evaluated. Because ELSD operates as a mass detector, the complex nature of in vitro-derived samples from hepatocyte incubations resulted in an inability to detect the analytes of interest in this matrix using ELSD. Additionally, the gradient nature of the analysis required to temporally separate ethoxycoumarin from its metabolites and matrix components interfered with the ELSD response. Furthermore, using less-complex contrived mixtures, ELSD demonstrated insufficient sensitivity (limit of detection of 1000–10,000 ng/mL) and an inconsistent inter-analyte response. Together, the limitations outlined in these experiments demonstrate that ELSD is at present an inadequate technique for generating semi-quantitative data on metabolites in drug discovery.

Keywords: Evaporative light-scattering detector; Metabolism; 7-Ethoxycoumarin; Metabolite identification; HPLC

1. Introduction

Elucidation of the routes of metabolism for a given drug candidate is an important component of the preclinical development process in the pharmaceutical industry. Definitive metabolite identification is typically initiated after a compound has been selected as a development candidate. Utilizing radiolabeled compound, detection and quantitation of metabolic routes via radiodetection is accomplished in these studies. However, due to several late-phase market withdrawals and advances in analytical instrumentation, preliminary metabolite identification is increasingly being initiated prior to clinical nomination [1]. The aim of these preliminary studies is to identify potential routes of metabolism, paying particular attention to indications of reactive intermediate formation. Such studies employ gradient LC/MS and LC/MS/MS, and have proven to be successful at identifying and characterizing in vitro routes of metabolism [2]. However, a major limitation of these studies is the requirement for radiolabeled drug substance for quantification of the amounts of the various metabolites formed. In a drug discovery setting, neither radiolabeled material nor analytical standards of drug metabolites are usually available. Unfortunately the ionization efficiency of even closely related molecules within the ion-source of a mass spectrometer differ, thus, simple metabolic modifications to a drug can drastically alter the mass spectral response, prohibiting comparison of mass spectrometrically derived peak areas to estimate abundance of the identified metabolites [3]. Furthermore, it is known that both molar absorptivity and the maximum absorption wavelength may be altered as a drug is metabolically modified, making the comparison of UV-derived peak areas without correction factors ambiguous [4].

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An ideal detector for metabolite identification work would not only be sensitive and selective (for qualitative detection), but would also deliver a universal response between metabolites and parent drug. The relative amounts of the metabolites could then be quantified accurately by calibrating the instrument with an external standard or the responses of the metabolites could be directly compared to the no-metabolism (known concentration) response of the parent drug (if operating within the linear dynamic range of the instrument). One proposed such "universal detector" is the evaporative light-scattering detector (ELSD). In ELSD, the analyte-containing mobile phase eluent from an HPLC is nebulized and evaporated, yielding a plume of analyte particles that are carried through the detector by a constant flow of nitrogen gas. The particles reflect and refract light, which is subsequently measured at a fixed angle from the incident light source. The extent of light scattering is proportional to the amount of analyte mass passing through the instrument [5]. The response should be essentially independent of the sample properties and hence structure of the analyte as long as the compound is not volatile and the analytes of interest do not substantially differ in molecular weight [5–8]. ELSDs have proven successful in the pharmaceutical industry and are routinely coupled with LC/MS instruments for screening of combinatorial libraries for chemical purity, using an external standard calibration curve to semi-quantitate the ELSD response [8].

Despite its widespread use in other applications, the ability of the ELSD to perform as a "universal" quantitative metabolite detector with an equivalent inter-analyte response for in vitro drug metabolism studies has not previously been explored. Therefore, the objective of the present work was to evaluate the performance of ELSD in the "universal" quantification of in vitro-derived metabolites. The goal was not to comprehensively profile the use of ELSD for this application, but to identify whether key limitations exist that would prohibit its use for this application. Due to the thorough characterization of its metabolic pathways [9], 7-ethoxycoumarin (EC) was selected as the analyte in these experiments.

2. Materials and methods

2.1. Chemicals

EC and 7-hydroxycoumarin (OHC) were purchased from Sigma Chemical Co. (St. Louis, MO). 7-Hydroxycoumarin sulfate (OHCS) was purchased from Ultrafine Ltd. (Manchester, UK). Acetonitrile (ACN), ammonium formate, formic acid, and dimethyl sulfoxide (DMSO) were all purchased from standard vendors and were of the highest available purity.

2.2. Hepatocytes

Fresh dog hepatocytes were purchased from CellzDirect (Tucson, AZ). Hepatocytes were suspended in Williams' Medium E with L-glutamine but without sodium bicarbonate or phenol red (JRH Biosciences, Lenexa, KS), and were used immediately upon delivery.

2.3. Instrumentation

All data were acquired through the UV and analog data channels of a Micromass Ultima quadrupole time of flight (Q-TOF) Mass Spectrometer (Waters, Milford, MA) operating Masslynx 4.0 software. Peak integration was completed utilizing the peak integration application in Masslynx's chromatogram view. HPLC was performed on an Agilent HP 1100 instrument (Agilent Technologies, Palo Alto, CA) with a diode array detector (DAD) and a PL-ELS 2100 ELSD (Polymer Laboratories Inc., Amherst, MA). Eluent flowed from the column through the DAD and into the ELSD. The DAD responses to EC, OHC, and OHCS were monitored at 322 nm. The optimized ELSD settings were determined to be 40 °C, 35 °C, and 0.8 standard liters per minute for the evaporator temperature, nebulizer temperature, and nebulizer gas flow rate, respectively. These values were optimized to maximize EC response by performing multiple injections of EC onto the system while independently stepping each parameter. The evaporator temperature was tested between 30 °C and 60 °C. The nebulizer temperature was tested between 30 °C and 45 °C. The gas flow rate was tested between 0.8 and 1.0 standard liters per minute. Approximately 80 psi of nitrogen gas was supplied to the ELSD gas inlet, and the output of the ELSD was a 1–V full-scale maximum analog signal as measured by the analog input channel on the Q-TOF. The signal was digitized by the analog-to-digital converter within the mass spectrometer. The analyte response delay between the DAD and ELSD varied from approximately 0.15 min to 0.25 min throughout this work.

2.4. Generation and analysis of test samples

The relative performance of UV detection and ELSD were first evaluated using in vitro metabolism samples generated in isolated hepatocytes. EC was incubated at 25 μM (approximately 4750 ng/mL) in a suspension of fresh dog hepatocytes (0.7×10^6 cells/mL) at 37 °C with gentle orbital shaking. The first sample aliquot was removed from the incubation at 5 min, and successive aliquots were removed every 30 min for 4 h. Metabolism was stopped by quick-freezing samples on dry ice. Hepatocyte incubates were thawed and centrifuged prior to analysis. An aliquot (200 μL) of the supernatant was injected onto a 25 cm × 4.6 mm (5 μ particle) BDS Hypersil C8 column (Thermo Electron, Bellefonte, PA) and eluted at 400 μL/min with a gradient that transitioned linearly from 2% to 100% ACN over 26 min. The column was selected to facilitate gradient elution of the analytes, based on previous experience in this laboratory.

Next, the ability of the ELSD to produce an equivalent response for EC and its metabolites was evaluated. Neat 1-mg/mL stock solutions of EC, OHC, and OHCS were pre-

pared in DMSO. A dilute mixture containing all three components at 10,000 ng/mL each was then prepared in H_2O. An aliquot (200 μl) of this mixture was injected (2000 ng on column) onto a 25 cm × 4.6 mm (5 μm particle) BDS Hypersil C8 column (Thermo Electron, Bellefonte, PA) and eluted at 400 μL/min with a gradient that transitioned linearly from 2% to 100% ACN over 26 min. Analyte elution was recorded via DAD and ELSD. Peak identity was verified by injecting separate samples that contained only one of the analytes each and monitoring retention time (data not shown).

To evaluate the influence of mobile phase composition on the ELSD response for EC, 100 μL of a 10,000-ng/mL solution of EC (made as described above) was injected (1000 ng on column) onto a 10 cm × 2.0 mm (3 μm particle) Luna Phenyl–Hexyl column (Phenomenex, Torrance, CA), selected to reduce overall analysis time in this mono-analyte experiment. Multiple injections were made with various fractions of ACN (40%, 50%, 70%, and 80%) contributing to the total flow rate of 400 μL/min, with the remainder of the flow being accounted for by the aqueous phase (10 mM ammonium formate, pH 3.0). Analyte elution was recorded via DAD and ELSD.

Finally, to exclude any influence of mobile phase composition on ELSD response, isocratic chromatography was investigated. One hundred microlitres of 10,000-ng/mL solutions of EC, OHC, and OHCS (made as described above) were each injected (1000 ng on column) onto a 5 cm × 3.0 mm (3 μm particle) Genesis C18 column (Jones Chromatography, Lakewood, CO), selected to rapidly elute the analytes onto the detector. 70% ACN/30% aqueous was maintained to elute the analytes from the column. Analyte elution was recorded via DAD and ELSD.

3. Results

3.1. Analysis of EC metabolism in hepatocytes

After optimization of the ELSD response to EC following successive injections (data not shown), the ELSD response was monitored for its ability to accommodate the types of chromatography and samples typical of a preliminary in vitro metabolic route assay. Incubates of EC in dog hepatocytes were injected onto the HPLC/UV/ELSD system and simultaneously monitored for UV and ELSD responses. The retention times of EC (∼25 min), OHC (∼21 min), and OHCS (∼18 min) were verified by UV detection of synthetically prepared standards (data not shown). UV and ELSD chromatograms of the 5-min incubate sample were substantially different (Fig. 1A and B). The UV chromatogram revealed detectable levels of all three analytes. The ELSD response, as expected, was more "universal", detecting many more of the components in this complex sample mixture. However, the ELSD chromatogram showed only a trace peak (180 peak area) for EC, with no detectable response for either OHC or OHCS. Similar results were observed following further metabolism; Fig. 1C and D show UV and ELSD chromatograms of the 30-min incubate sample. The UV chromatogram revealed all three analytes, and indicated that approximately 35% of the EC had been metabolized and that the concentrations of both metabolites increased from the levels observed at 5 min. However, even after further metabolism, none of the analytes were detectable via ELSD in the 30-min sample (Fig. 1D).

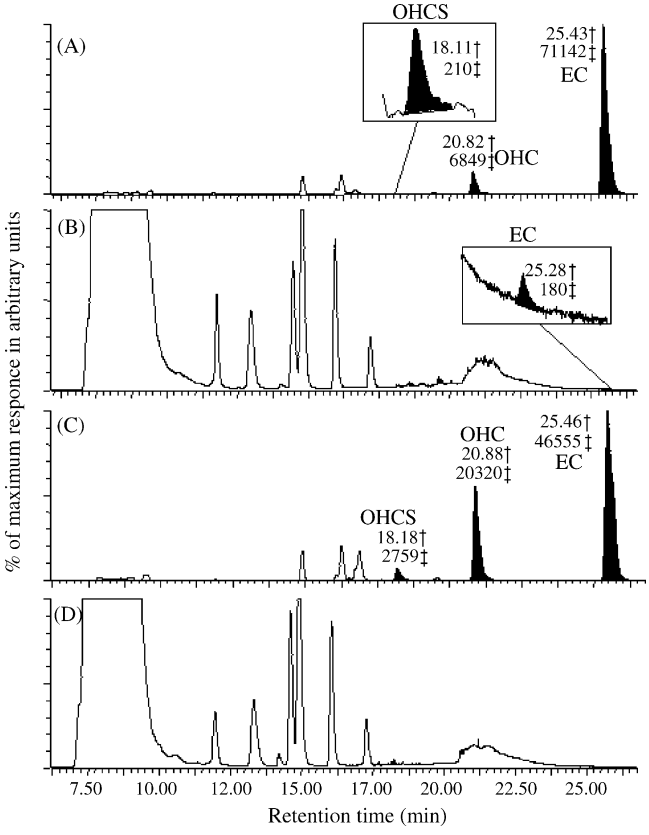

Fig. 1. UV chromatograms (extracted at 322 nm (A and C)) and ELSD chromatograms (B and D) of 7-ethoxycoumarin (∼25 min), 7-hydroxycoumarin (∼21 min), and 7-hydroxycoumarin sulphate (∼18 min) in dog hepatocyte incubate samples incubated for 5 (A and B) or 30 (C and D) min. †, Retention time (min), ‡, peak area in arbitrary units.

The UV intensities of EC and its OHC and OHCS metabolites in this experiment were consistent with those typically observed in this laboratory. Although the UV response continued to monitor the complete metabolism of EC and increase in metabolite concentration over the course of the 4-h incubation, the ELSD was never capable of detecting any of the metabolites (data not shown). To further characterize potential reasons for the inability of the ELSD to detect metabolites in hepatocyte incubate samples, various additional experiments were performed.

3.2. Gradient analysis of a neat mixture of EC, OHC, and OHCS

As the ELSD did not exhibit a sensitive response to EC and its metabolites in a complex in vitro matrix, its re-

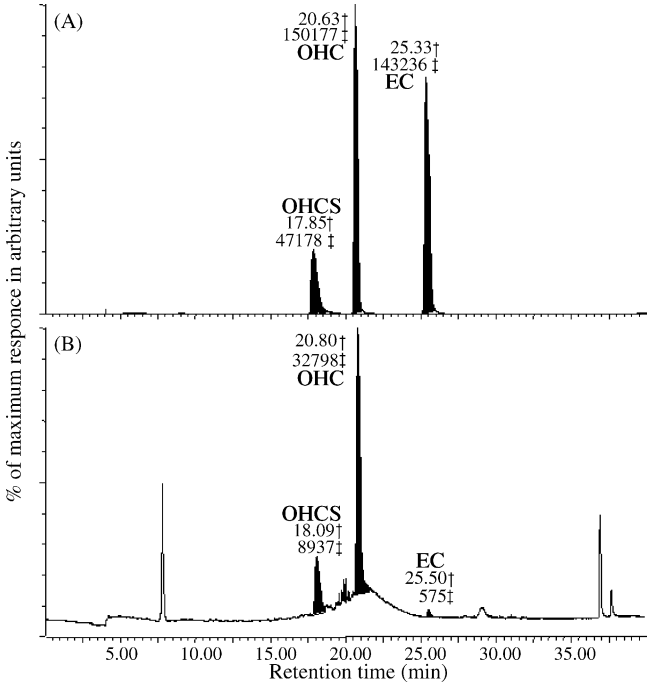

Fig. 2. UV (extracted at 322 nm (A)) and ELSD chromatograms (B) of a neat 10,000-ng/mL mixture of EC (∼25 min), OHC (∼21 min), and OHCS (∼18 min). †, Retention time (min), ‡, peak area in arbitrary units.

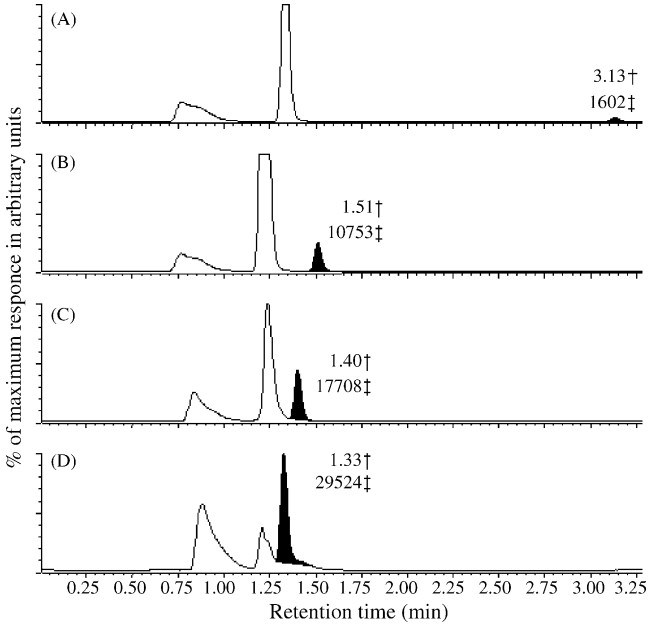

Fig. 3. ELSD chromatograms of neat 10,000 ng/mL 7-ethoxycoumarin using an isocratic elution containing 40% (A), 50% (B), 70% (C), or 80% (D) acetonitrile. †, Retention time (min), ‡, peak area in arbitrary units.

sponse to a simpler, chemically-contrived system was monitored. Response to a neat synthetically prepared mixture of EC, OHC, and OHCS at 10,000 ng/mL in water was measured using the same gradient LC conditions described above (Fig. 2). Prepared at equimass concentrations, all three components were detected with both detectors but with non-uniform intensities. The UV response showed peak areas for OHC and OHCS of 105% and 33% of the EC peak area, respectively (Fig. 2A). Due to likely differences in absorption wavelength and molar absorptivity, uniform UV detection was not expected, furthermore, the UV response is derived from the number of molecules detected, not the mass [10]. In this experiment, the trend of the UV response did follow the molar concentration when corrected for the molecular weights of the three analytes. However, without knowledge of molar absorptivities, as is typically the situation when performing preliminary metabolite identification, this trend is not useful for metabolite quantification. Evaluation of the ELSD signal for the same sample (Fig. 2B) revealed an even less uniform response. At the same 10,000 ng/mL concentration in water, the ELSD peak areas for OHC and OHCS were 57 and 16 times that of EC, respectively.

3.3. Effect of mobile phase composition on ELSD response

It has previously been claimed that gradient composition can influence the ELSD response [7]. Therefore, the effect of mobile phase composition was studied by performing neat injections of 10,000 ng/mL EC with isocratic elutions of various acetonitrile concentration and detection via UV and ELSD. As the fraction of ACN was varied from 40% to 80% the UV response decreased approximately 40% (data not shown), while over the same range the ELSD response increased nearly 20-fold (Fig. 3). The means of the peak areas collected using the multiple isocratic conditions were calculated for the UV and ELSD responses; the standard deviations for the UV and ELSD responses were 20% and 79%, respectively of the mean peak area for each detection method. Similar deviations in response were observed for OHC and OHCS over the same range of ACN composition (data not shown). Throughout the course of a gradient, the composition of ACN in the mobile phase is continually changing by definition. These results, which demonstrated a significant influence of the composition of the mobile phase on the ELSD response to EC, indicated that quantitation of EC and its metabolites via gradient HPLC with ELSD is not possible.

3.4. ELSD analysis with isocratic chromatography

Due to the variability in ELSD response under gradient LC conditions, its response to EC, OHC, and OHCS under identical isocratic LC conditions was investigated. Neat synthetically prepared standards (10,000 ng/mL) of each compound were analyzed separately with a mobile phase composition of 70/30% ACN/aqueous (Table 1). Neither detector provided an equivalent analyte response for EC, OHC, or OHCS. Using UV detection, the integrated peak areas of OHC and OHCS were 147% and 51% of the peak area of EC, respectively. For ELSD, the integrated peak areas for OHC

Table 1
Comparison of ELSD and UV peak area responses with isocratic chromatography of neat 10,000 ng/mL solutions

Analyte	ELSD peak area (arbitrary units)	UV peak area at 322 nm (arbitrary units)	Percentage of EC ELSD peak area	Percentage of EC UV peak area
EC	8343	495,922	100	100
OHC	49,930	728,399	598	147
OHCS	33,803	252,457	405	51

and OHCS were 598% and 405% of the peak area of EC, respectively. These results suggest that even under isocratic conditions ELSD response to these analytes at 10,000 ng/mL is not uniform.

4. Discussion

During drug discovery, routes of metabolism for a lead molecule or chemical series are often of interest, both to screen for evidence of reactive metabolite pathways and to help guide iterative medicinal chemistry efforts to improve metabolic stability [11]. In either instance, an estimate of amounts of various metabolites formed would be very useful in interpreting these studies. For drug discovery purposes, even values within twofold of the actual concentration of metabolite formed would be useful in guiding lead optimization. Current analytical techniques do not allow this degree of quantitative accuracy for the various reasons noted in Section 1, therefore, alternative techniques are actively under investigation to meet this analytical need.

The success of ELSD in applications such as purity screening of combinatorial libraries is well-documented [8,10]. In such applications, ELSD is often used to quantify concentrations of analytes in the μM to mM concentration range, with quantitation errors of as little as ±10% using external instrument calibration standards [8,10]. Therefore, ELSD seemed a reasonable approach to attempt to quantify metabolites from a typical in vitro experiment. Unfortunately, during this investigation, several limitations of the ELSD approach to metabolite quantification became manifest. Upon first evaluation, it became evident that the complex nature of the hepatocyte incubation medium, coupled with the relatively low (25 μM) starting analyte concentration, was not compatible with the use of ELSD. Because of the inherent non-selective detection abilities of the ELSD [8], interference with the analyte by other components in the hepatocyte incubation mixture will be unavoidable, although it is possible that these interferences could be further minimized with exhaustive sample clean-up procedures such as solid phase extraction. However, the low sensitivity of ELSD in this application will be difficult to circumvent, as solubility limitations coupled with the need to minimize organic content in metabolism incubation mixtures will limit the maximum concentration of most xenobiotics [12].

In addition to the difficulties encountered in the hepatocyte incubation samples, limitations to the ELSD quantitation approach were also evident in more contrived, less complex, systems. Upon removal of the complex matrix, although the key analytes of interest were detectable, consistent inter-analyte peak area intensity was not achievable regardless of the manipulation employed. As anticipated based on a previous report [7], the gradient nature of the analysis was at least in part responsible for this differential response. Although gradient HPLC analysis would be anticipated to be required for the analysis of experimental samples (to temporally separate parent drug from metabolites), some ELSD applications could be envisioned where an isocratic condition would be suitable. Ultimately, however, at relevant concentrations for drug metabolism studies, ELSD did not provide a sufficiently consistent inter-analyte response, even under isocratic conditions, to be used for quantitation of drug metabolites. Furthermore, even in relatively simple matrices, the sensitivity of ELSD for the analytes in this study was inadequate for this purpose, ranging from 1000 to 10,000 ng/mL (data not shown).

Despite the limitations of ELSD outlined here, future technical advances in sensitivity and/or response uniformity may well render this technique valuable for the drug metabolism application. Further, additional non-traditional techniques for metabolite quantification are under investigation in this laboratory. For example, chemiluminescent nitrogen detection has previously been shown to have promise in the field of metabolite quantification [3,13], and will be explored in further detail in future experiments.

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