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Simultaneous determination of 1-chloro-2,4-dinitrobenzene, 2,4-dinitrophenyl-*S*-glutathione and its metabolites for human placental disposition studies by high-performance liquid chromatography

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Abstract

We developed and validated an HPLC method for determination of 1-chloro-2,4-dinitrobenzene (CDNB) and its glutathione conjugate 2,4-dinitrophenyl-*S*-glutathione (DNP-SG) to study the kinetics and mechanisms involved in DNP-SG formation and efflux, as a probe for human placental metabolism and transport. This method combines use of 3 μm solid phase, rapid mobile phase gradient with dual wavelength ultraviolet detection to permit determination of a lipophilic parent compound and its hydrophilic metabolites in a single short run. The selectivity, linearity, accuracy, precision, relative recovery and stability of the assay are sufficient for determining CDNB, DNP-SG and its metabolites from buffer and tissue samples to support placental drug metabolism and transport studies.
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Keywords: 1-Chloro-2,4-dinitrobenzene (CDNB); 2,4-Dinitrophenyl-*S*-glutathione (DNP-SG); Placental metabolism and transport; GSTP1-1; ABCC2; ABCG2

1. Introduction

1-Chloro-2,4-dinitrobenzene (CDNB) is a toxic xenobiotic known to cause oxidative stress and cell death. Human placental glutathione-*S*-transferase isoform P1-1 (GSTP1-1) conjugates CDNB with glutathione to form 2,4-dinitrophenyl-*S*-glutathione (DNP-SG) [1]. DNP-SG is a known substrate of ATP-binding cassette (ABC) transporter isoforms C1 and C2 (multidrug resistance associated proteins 1 and 2) [2] and isoform G2 (breast cancer resistance protein) [3]. In the human placenta ABCC2 and ABCG2 are located on the apical membrane of the syncytiotrophoblast layer and may be involved in protecting the fetus from xenobiotic exposure. ABCC1 is located on the basolateral membrane of the syncytiotrophoblast layer and on the albuminal side of the fetal capillary endothelium and may be involved in preventing the accumulation of toxic xenobiotics in the syncytiotrophoblast or the excretion of fetal waste products and prevention of fetal exposure to xenobiotics that cross the syncytiotrophoblast [4]. The role of these transporters in the efflux of xenobiotics as well as endogenous substances in different organs such as the liver, brain, intestine and kidneys is well established [5]. However, their role in the placental handling of drugs or endogenous substances is not clearly understood.

The paradigm of glutathione conjugation of CDNB followed by DNP-SG efflux has been widely used to study the coordinated glutathione conjugation and MRP transport functions in several biological systems including the rat or guinea pig liver [6–13] and intestine [14–17], human erythrocytes [18], human colon adenocarcinoma cells (Caco-2) [19,20], human IGR-39 melanoma cells [21] and MCF7 breast cancer cells [22]. Mrp2-

Abbreviations: ABC, ATP-binding cassette; ABCC1, ABC transporter isoform C1; ABCC2, ABC transporter isoform C2; ABCG2, ABC transporter isoform G2; CDNB, 1-chloro-2,4-dinitrobenzene; DNP, 2,4-dinitrophenol; DNP-CG, 2,4 dinitrophenyl-*S*-cysteinylglycine; DNP-Cys, 2,4-dinitropheyl-*S*-cysteine; DNP-NAc, 2,4-dinitrophenyl-*N*-acetylcysteine; DPBS, Dulbecco's phosphate-buffered saline; FDNB, 1-fluoro-2,4-dinitrobenzene; GSTP1-1, glutathione-*S*-transferase isoform P1-1; MRP, multidrug resistance associated protein (human isoform); Mrp, multidrug resistance associated protein (rat isoform); PCA, perchloric acid

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mediated biliary efflux of DNP-SG is extremely efficient and has been well characterized in the isolated rat liver perfusion model [12] and the approach has been extended to the characterization of endocytic retrieval of Mrp2 under conditions of cholestasis induced by agents such as estradiol-17β-D-glucuronide [9], ethinylestradiol [6], *t*-butyl hydroperoxide and CDNB itself [10]. Mrp2-mediated intestinal DNP-SG efflux has also been established [14], and has been studied under conditions of hyperbilirubinemia [17] as well as in postpartum rats [15] and partially hepatectomized rats [16].

This model is analytically convenient since DNP-SG (λ_{max} = 340 nm) and CDNB (λ_{max} = 280 nm) can be readily distinguished spectrophotometrically. However, in ex vivo or in vivo systems, DNP-SG may undergo further metabolism by the action of γ-glutamyl transferase or dipeptidase to form DNP-CG or DNP-Cys, respectively. DNP-Cys is a precursor in the *N*-acetyltransferase-mediated formation of DNP-NAc as a part of the mercapturic acid pathway [8]. The activity of human placental GSTP1-1 towards CDNB metabolism has been described [1]; however the efflux mechanism for DNP-SG from human placental tissue has not been established. Furthermore, formation of secondary metabolites of DNP-SG might be expected since γ-glutamyl transferase [23] and *N*-acetyltransferase [24] activities have been detected in the human placenta.

Methods are available for the analysis of DNP-SG from various biological matrices using high-performance liquid chromatography (HPLC) coupled with ultraviolet detection [8,17,19,21,25]. Simple spectrophotometric absorbance measurements at 335–340 nm have also been reported for DNP-SG quantitation [9,12,18]; however, CDNB as well as the metabolites of DNP-SG have some absorbance at 340 nm and may interfere the quantitation. Hinchman et al. [8] reported a HPLC method with ultraviolet detection for simultaneous determination of DNP-CG, DNP-Cys, DNP-SG and DNP-NAc at 365 nm from bile, perfusate and liver tissue in the rat liver perfusion model. The method employed isocratic elution with 25% acetonitrile and 0.1% phosphoric acid in water and has been used in several studies [6,14,16]. This method does not enable quantitation of CDNB and the lower limit of quantitation has not been reported. Yokooji et al. [17] reported separate HPLC-UV methods involving isocratic elution with acetonitrile and 1% acetic acid in water (15:85, v/v) for DNP-SG detection at 365 nm and elution with acetonitrile and 1% acetic acid in water (35:65, v/v) for CDNB detection at 305 nm. Gradient elution methods have also been reported for the simultaneous detection of DNP-SG and DNP-NAc [19,21]. However, most of the reported methods have not been validated and do not enable simultaneous, rapid detection and quantification of CDNB, DNP-SG and its metabolites. Additionally DNP may be formed via hydrolysis of CDNB but was not analyzed in any of the reported assays.

The present study describes a method for the simultaneous, rapid detection of CDNB and DNP-conjugates from human placental tissue homogenate and Dulbecco's phosphate-buffered saline (DPBS). The salient features of this method include simple pretreatment, improved selectivity, short run time and simultaneous detection of CDNB, DNP, DNP-SG and its metabolites DNP-Cys and DNP-NAc. The method was validated in both matrices and was applied to the quantitation of CDNB, DNP-SG and its metabolites from placental tissue homogenate and DPBS upon exposure of human placental villous tissue fragments to CDNB, to support human placental drug transport studies.

2. Experimental

2.1. Chemicals and reagents

CDNB (99% purity), *N*-acetyl-L-cysteine (98% purity) and perchloric acid (PCA) reagent (70% in water) were purchased from Acros Organics (Morris Plains, NJ, USA). L-Glutathione reduced; GSH (99% purity) and cysteinylglycine (85% purity) were purchased from Sigma–Aldrich (St. Louis, MO, USA). 1-Fluoro-2,4-dinitrobenzene; FDNB (99% purity) was from Fluka Biochemika (Buchs, Switzerland). L-Cysteine and 2,4-dinitrophenol (90–95% purity) were purchased from MP Biomedicals Inc. (Solon, OH, USA). HPLC grade glacial acetic acid and acetonitrile were purchased from J.T. Baker Chemical Co. (Phillipsburg, NJ, USA) and Fisher Chemicals (Fair Lawn, NJ, USA), respectively. Water was filtered through the NANOpure Diamond Ultrapure Water system from Barnstead International (Dubuque, IA, USA). All other chemicals, solvents or reagents were of analytical grade and were obtained from Sigma–Aldrich or Fisher Chemicals unless indicated below.

2.2. Chemical synthesis of HPLC standards

Reference standards for the 2,4-dinitrophenyl conjugates of glutathione, cysteine, *N*-acetylcysteine and cysteinylglycine are commercially unavailable and were synthesized as reported [8]. FDNB was used as the derivatizing agent. Briefly, FDNB (0.01 mol in 10 mL methanol) was incubated with reduced L-glutathione, L-cysteine hydrochloride, *N*-acetyl-L-cysteine or cysteinylglycine (0.01 mol in 1–2N potassium carbonate) at room temperature for 15 min with stirring, filtered, acidified to pH 2 with dilute HCl and the resulting compounds were recrystallized from boiling water in the form of crystals of DNP-SG, DNP-Cys, DNP-NAc or DNP-CG, respectively. The yield of DNP-CG after recrystallization was poor, it was not detected in preliminary studies, and the compound underwent degradation upon storage; therefore it was not further analyzed.

Identity of the synthesized standards was determined by time of flight mass spectrometry in the positive ion mode on a Q-tof micromass spectrometer (Waters, Milford, MA, USA). Peak purity was monitored by the HPLC method, as explained below after individual injections of standards, followed by ultraviolet detection at 340 or 280 nm. Concentrations of standards in the stock solutions were determined spectrophotometrically by measuring absorbance in water at 340 nm on a UV-1700 PharmaSpec Spectrophotometer (Shimadzu, Japan) and using previously reported molar extinction coefficients [8]. The stock solutions were diluted to 500 μM working solutions in 10% perchloric acid in water. The stock solutions and working solutions were stored at −20 °C.

2.3. Preparation of assay standard samples

2.3.1. Standards of DNP-conjugates and CDNB in buffer

Standard samples of DNP-Cys (0.1, 0.2, 1, 4, 20, 50 μM), DNP-SG (0.1, 0.2, 1, 4, 20, 100 μM), DNP-NAc (0.25, 0.5, 1, 2, 4, 20 μM), DNP (1, 1.5, 2, 4, 10, 20 μM) and CDNB (1, 1.5, 2, 4, 10, 20 μM) were prepared by spiking 500 μL buffer, Dulbecco's phosphate-buffered saline (DPBS) [calcium chloride 100 mg/L, magnesium chloride 100 mg/L, potassium chloride 200 mg/L, potassium phosphate monobasic 200 mg/L, sodium chloride 8 g/L, sodium phosphate dibasic 2.16 g/L, D-glucose 1 g/L and sodium pyruvate 36 mg/L in water, pH 7.4] with appropriate volumes of 500 μM working solutions prepared as described above and treated with 10% PCA in water (1:1, v/v) to a final volume of 1 mL. Quality control (QC) samples containing DNP-Cys (0.3, 1, 40 μM), DNP-SG (0.25, 6, 80 μM), DNP-NAc (0.6, 2, 16 μM), DNP (2, 4, 16 μM) and CDNB (2, 4, 16 μM) were independently prepared in the same manner. One hundred microliters of the standard or QC sample was injected into the HPLC for quantitation.

2.3.2. Standards of DNP-conjugates and CDNB in human placental tissue homogenate

Representative normal human placental tissue stored at −80 °C was thawed and homogenized in 1:10 (w/v) buffer (50 mM Tris, 1 mM EDTA, 1 mM PMSF, 5 μg/mL aprotinin, 5 μg/mL leupeptin, 2 μg/mL pepstatin A, 2 μg/mL antipain, 1 mg/mL soya trypsin inhibitor, pH 7.4 at 4 °C) using a Polytron PT 10–35 homogenizer with a PTA 10 TS generator (Kinematica, Lucerne, Switzerland; speed setting 6.5), for 30 s on ice. Tissue homogenate samples were centrifuged at 6000 × *g* for 5 min at 4 °C, 500 μL aliquots were snap frozen by dropping into liquid nitrogen and stored at −80 °C.

Standards and QC samples containing DNP-Cys, DNP-SG, DNP-NAc, DNP and CDNB at the same concentrations as the buffer standards and QC samples, respectively, were prepared by spiking 500 μL of tissue homogenate with appropriate amounts of 500 μM working solutions, treated with 10% PCA (1:1, v/v) to a final volume of 1 mL and centrifuged at 10,000 rpm for 5 min at 4 °C. One hundred microliters of the supernatant was injected into the HPLC system for quantitation.

2.4. Instrumentation

Separation of analytes was performed using an Alltima HP C18 (Alltech, Deerfield, IL, USA) column (100 mm × 4.6 mm × 3 μm) preceded by a POLAR-RP (4 mm × 3.0 mm) security guard cartridge (Phenomenex, Torrance, CA, USA). The HPLC system (Waters) consisted of the Alliance 2695 separations module and the 2487 dual wavelength ultraviolet absorbance detector. Data were collected and analyzed by the Empower 2 chromatography data software (Waters).

Table 1
Gradient elution

Time (min)	A (%)	B (%)
0	80	20
2	80	20
4	70	30
6	30	70
8	30	70
10	80	20

A is 1% acetic acid in water and B is acetonitrile.

2.5. Chromatographic conditions

Determination of DNP-conjugates and CDNB was performed with gradient elution as shown in Table 1 at a flow rate of 1 mL/min. The mobile phase A was 1% acetic acid in water (pH 2.8) while mobile phase B was 100% acetonitrile. The column temperature was maintained at 30 °C and the autosampler compartment was set to maintain sample temperature at 4 °C. The injection volume was 100 μL. DNP-Cys, DNP-SG and DNP-NAc were detected at 340 nm while, DNP and CDNB were detected at 280 nm.

2.6. Validation

The linearity of the assay for each of the DNP-conjugates and CDNB, in both buffer and tissue homogenate was assessed by analyzing peak areas for calibration standards (1/*C* weighted) of DNP-Cys, DNP-SG, DNP-NAc, DNP and CDNB in the concentration range of 0.1–50, 0.1–100, 0.25–20, 1–20 and 1–20 μM, respectively. The intraday accuracy and precision were evaluated by assaying three replicates of the QC samples each in two analytical runs on the same day. The interday accuracy and precision were evaluated by assaying three replicates of the QC samples each in analytical runs on 3 different days. Precision was characterized by the coefficients of variance (CV, %) whereas accuracy was expressed as deviation from the nominal value (DFN, %). Recovery of analytes from the tissue homogenate was assessed by comparing peak areas of the extracted QC samples with the peak areas of QC samples prepared in homogenizing buffer, and was expressed as a percentage area of the extracted QC sample relative to that of the corresponding QC sample in homogenization buffer. Short-term storage stability was evaluated over a period of 2 weeks by freezing spiked buffer QC samples at −20 °C or spiked tissue homogenate QC samples at −80 °C, quantifying the analytes at 0, 24, 48, 96 h, 1 week and 2 weeks after thawing the samples at room temperature and comparing the concentrations with the concentrations analyzed at time 0.

2.7. Application

The HPLC method was applied to the quantitation of DNP-conjugates and CDNB from buffer and human placental tissue homogenates to support placental drug transport studies. The study was approved by the VCU Institutional Review Board (IRB) and informed consent was obtained from the patients prior

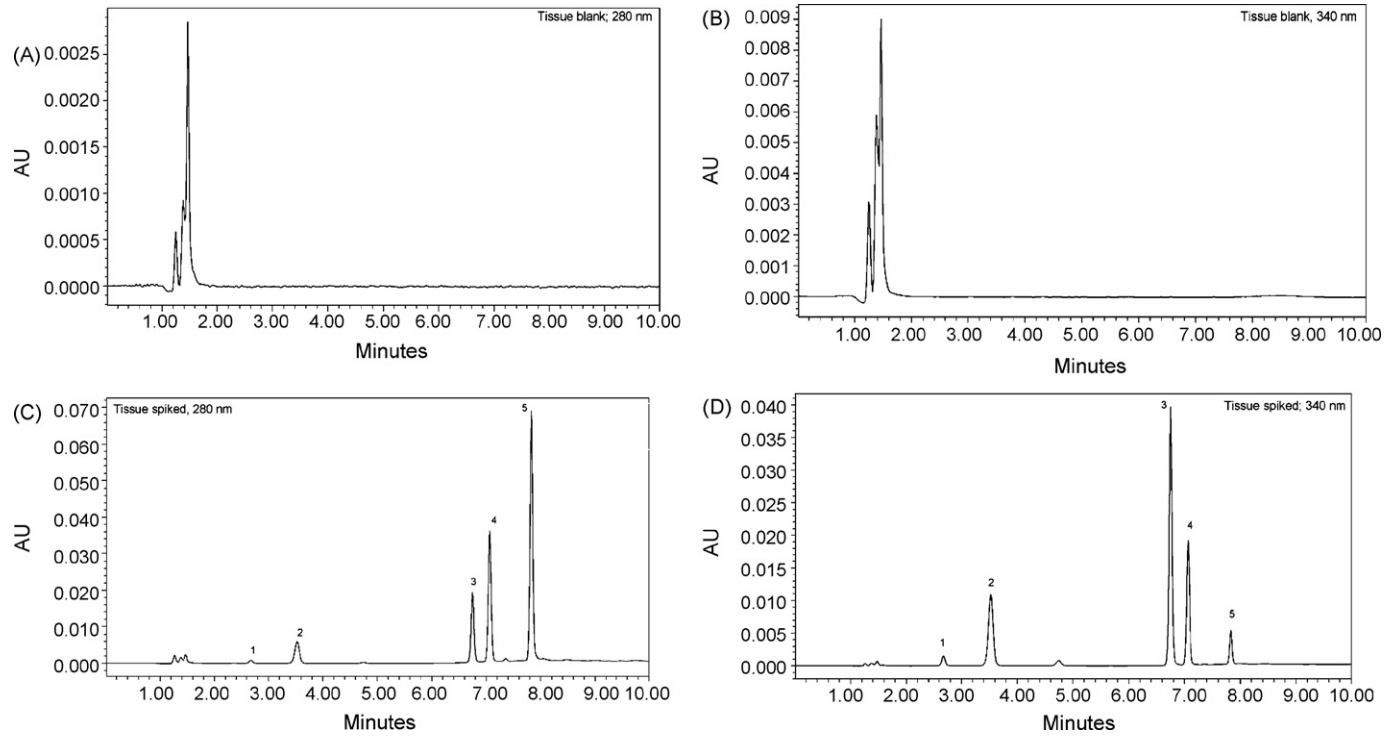

Fig. 1. Representative chromatograms for blank tissue homogenate at 280 nm (A) and 340 nm (B), and tissue homogenate spiked with 10 μM of each of the analytes, DNP-Cys (1), DNP-SG (2), DNP-NAc (3), DNP (4) and CDNB (5) at 280 nm (C) and 340 nm (D).

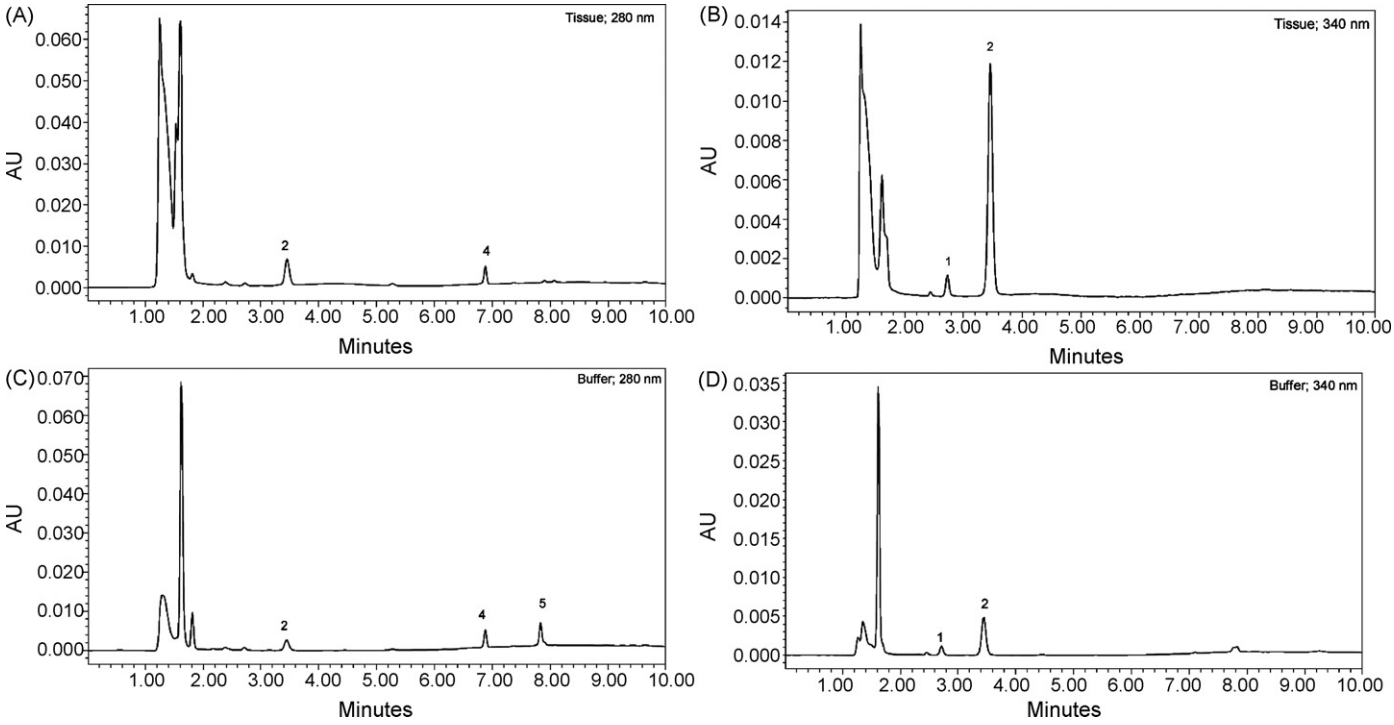

Fig. 2. Representative chromatograms for detection of DNP-Cys (1) and DNP-SG (2) at 340 nm and DNP (4) and CDNB (5) at 280 nm from experimental placental tissue homogenate and buffer samples after brief exposure to 100 μM CDNB followed by incubation with DPBS at 37 °C for 20 min. Tissue DNP-Cys (1) and DNP-SG (2) concentrations were 0.18 and 2.6 μM, respectively (B) and buffer DNP-Cys (1) and DNP-SG (2) concentrations were 0.15 and 1.4 μM, respectively (D). Tissue and buffer DNP (4) and CDNB (5) concentrations were below the LLOQ (A and C). DNP-NAc was not detected. Sample volume = 100 μL.

to delivery. Placental tissue samples were obtained from women (between ages of 18 and 45, gestational length ≥36 weeks) within 30 min of birth from cesarean section deliveries following normal pregnancies in three patients at the VCU Medical Center Hospital. Minced villous tissue was cultured in M199 medium with 10% FBS for 24 h to enable pretreatment with inhibitors of enzyme or transporter function. Villous tissue (200 mg) was preincubated without or with 5 mM ATPase inhibitor sodium orthovanadate, then exposed to 100 μM CDNB for 5 min at 10 °C, rinsed twice in DPBS and incubated with shaking at 200 rpm in DPBS without or with 500 μM sodium orthovanadate at 37 °C for varying time intervals in 75 μm net wells in 12-well culture dishes. Tissue and 200 μL buffer samples were collected in 200 μL 10% PCA in water on ice at 5, 10, 20, 45 min and stored at −80 and −20 °C, respectively. Tissue was homogenized on ice in 3 mL of Tris–EDTA buffer as described above and centrifuged at 6000 × *g* for 5 min at 4 °C. The supernatant was treated with 10% PCA in water (1:1, v/v), centrifuged at 10,000 × *g* for 5 min at 4 °C and DNP-conjugates and CDNB in 100 μL of the PCA treated tissue homogenate supernatant or buffer was assayed by HPLC. The effect of sodium orthovanadate treatment on the efflux of DNP-SG from villous tissue fragments upon 20 min DPBS incubation at 37 °C was compared using a paired Student's *t*-test using Prism version 4 (GraphPad Software Inc., San Diego, CA, USA).

3. Results and discussion

3.1. Analysis of HPLC standards

The identity of the synthesized DNP-conjugates was established using mass spectrometry. Time of flight mass spectra for DNP-Cys, DNP-SG and DNP-NAc in the positive ion mode showed prominent fragment ions at *m*/*z* ratios of 288.01, 473.7 and 330.02, respectively, consistent with their expected molecular weights (287.2, 473.4 and 329.3, respectively). Single peaks were observed by UV detection at both 340 and 280 nm at mean peak retention times of 2.72, 3.44 and 6.61 min after individual injections of synthesized standards of DNP-Cys, DNP-SG and DNP-NAc, respectively. No impurity peaks were observed and the peak purity was greater than 99%. Concentrations of the synthesized standards in stock solutions were estimated using molar extinction coefficients reported by Hinchman et al. [8] and by measuring absorbance in water at 340 nm.

3.2. Chromatographic conditions

The difference in the hydrophobicity of DNP-SG (cLogD at pH 7.0 = 2.06; ACD/LogD v8.14) and CDNB (cLogD at pH 7.0 = −2.85; ACD/LogD v8.14) posed a problem in the simultaneous detection of these analytes under isocratic conditions. The use of the Alltima C18 column with 3 μm particle diameter under gradient elution conditions enabled simultaneous, rapid detection of five analytes within 8 min with acceptable resolution. DNP-Cys DNP-SG, and DNP-NAc were detected at 340 nm at 2.72, 3.47 and 6.61 min, respectively, while DNP and CDNB were detected at 280 nm at 6.95 and 7.84 min, respectively. The use of a dual wavelength UV detector enhanced the selectivity of detection for DNP and CDNB, since these analytes were found to have a maximum spectral absorbance at 280 nm in the mobile phase. The extraction procedure was consistent with previously reported methods and involved treatment with 10% PCA in water (1:1, v/v) for protein precipitation from the tissue homogenate and buffer samples. The method offers the advantages of greater selectivity, a shorter run time and simultaneous detection of CDNB, DNP-SG and its metabolites from both DPBS as well as human placental tissue homogenate samples following a simple pretreatment with 10% PCA in water. Furthermore, inhibitors used for metabolism and transport studies such as ethacrynic acid, MK571, verapamil, dipyridamole, probenecid, sulfinpyrazone and bromosulfophthalein were either not detected or had retention times different from the those of the DNP-conjugates or CDNB and did not interfere with the analytical method (unpublished results).

The typical chromatograms for blank tissue homogenate at 280 nm (A) and 340 nm (B) and tissue homogenate spiked with 10 μM of each of the analytes at 280 nm (C) and 340 nm (D) are presented in Fig. 1. Representative chromatograms illustrating the application of this method for detection of tissue synthesis and subsequent efflux of DNP-SG and DNP-cys into the buffer are presented in Fig. 2. Chromatograms for the tissue homogenate and buffer samples were obtained at 280 nm (A and C) and at 340 nm (B and D) following incubation of 200 mg human placental villous tissue with 100 μM CDNB in DPBS at 10 °C for 5 min and a further 20 min incubation in DPBS at 37 °C. The total run time for the assay in each of the matrices was 10 min.

3.3. Buffer assay validation

The retention time, LLOQ and regression coefficients for the analysis of DNP-Cys, DNP-SG, DNP-NAc, DNP and CDNB from DPBS for $n = 6$ analytical runs are summarized in Table 2. The intraday and interday results for accuracy and precision at the LLOQ and the QC concentrations are presented in Table 3. For the QC samples, both interday and intraday accuracy showed less than 11.57% DFN (except QC1 for DNP-NAc) and the precision was within 16.51% CV, for all the analytes. There was a good linear relationship (1/*C* weighted) between peak areas and $C(x)$ over the concentration range of 0.1–50 μM for DNP-Cys with linear regression yielding $y = (12612 \pm 43.4)x + (15.95 \pm 0.36)$, 0.1–100 μM for DNP-SG $[y = (-517.7 \pm 18.93)x + (19.87 \pm 0.03)]$, 0.25–20 μM for DNP-NAc $[y = (-471.3 \pm 90.02)x + (25.86 \pm 0.61)]$ and 1–20 μM for both DNP $[y = (-141.6 \pm 49.73)x + (6.52 \pm 0.012)]$ and CDNB $[y = (426.5 \pm 49.64)x + (7.83 \pm 0.01)]$.

3.4. Tissue homogenate assay validation

The retention time, LLOQ and regression coefficients for the analysis of DNP-Cys, DNP-SG, DNP-NAc, DNP and CDNB from human placental tissue homogenate for $n = 6$ analytical runs are summarized in Table 4. The intraday and

Table 2
Assay parameters for determination of analytes from DPBS

Analyte	Concentration range (μM)	Retention time[a] (min)	LLOQ (μM)	Regression coefficient[b]
DNP-Cys	0.1–50	2.72 ± 0.005	0.1	0.9861–0.9917
DNP-SG	0.1–100	3.47 ± 0.004	0.1	0.9998–0.9999
DNP-NAc	0.25–20	6.61 ± 0.059	0.25	0.9973–0.9999
DNP	1.0–20	6.95 ± 0.026	1.0	0.9994–0.9999
CDNB	1.0–20	7.84 ± 0.007	1.0	0.9991–0.9998

LLOQ, Lower limit of quantification; sample volume = 100 μL.
[a] Mean ± S.D. for six replicates.
[b] Range.

Table 3
Assay validation results for determination of analytes from DPBS

Sample	Nominal concentration (μM)	Intraday ($n = 6$)			Interday ($n = 9$)		
		Measured concentration (μM)	Precision CV (%)	Accuracy DFN (%)	Measured concentration (μM)	Precision CV (%)	Accuracy DFN (%)
DNP-Cys							
LLOQ	0.1	0.108 ± 0.021	19.44	8.02	0.089 ± 0.017	19.12	11.11
QC1	0.3	0.284 ± 0.047	16.51	−5.14	0.301 ± 0.045	14.82	0.18
QC2	10	10.8 ± 0.77	7.20	7.63	9.73 ± 0.94	9.61	−2.67
QC3	40	38.9 ± 2.17	5.57	−2.76	37.0 ± 3.39	9.18	−7.56
DNP-SG							
LLOQ	0.1	0.118 ± 0.021	17.80	18.03	0.093 ± 0.017	18.28	−17.80
QC1	0.25	0.233 ± 0.020	8.64	−6.75	0.221 ± 0.028	12.63	−11.57
QC2	6	5.81 ± 0.09	1.59	−3.17	5.80 ± 0.07	1.20	−3.36
QC3	80	81.8 ± 0.31	0.37	2.25	83.5 ± 4.08	4.89	4.33
DNP-NAc							
LLOQ	0.25	0.254 ± 0.009	3.54	1.60	0.262 ± 0.041	15.64	4.80
QC1	0.6	0.724 ± 0.008	1.12	20.62	0.717 ± 0.016	2.24	19.55
QC2	2	2.06 ± 0.03	1.20	2.96	1.80 ± 0.19	10.35	−9.97
QC3	16	15.6 ± 0.12	0.77	−2.25	14.8 ± 0.64	4.33	−7.41
DNP							
LLOQ	1	1.09 ± 0.05	4.59	9.00	1.03 ± 0.03	2.91	3.00
QC1	2	2.00 ± 0.03	1.57	0.09	1.93 ± 0.06	2.96	−3.67
QC2	4	4.05 ± 0.07	1.84	1.24	4.11 ± 0.07	1.70	2.63
QC3	16	16.0 ± 0.25	1.58	−0.09	16.6 ± 0.64	3.85	3.86
CDNB							
LLOQ	1	1.03 ± 0.02	1.94	2.91	1.04 ± 0.03	2.88	3.85
QC1	2	2.01 ± 0.05	2.48	0.61	1.99 ± 0.02	1.02	−0.60
QC2	4	4.05 ± 0.08	1.94	1.26	4.01 ± 0.07	1.69	−0.01
QC3	16	15.9 ± 0.38	2.38	−0.76	15.6 ± 0.34	2.16	−2.07

Intraday data based on triplicate determinations in two analytical runs on the same day. Interday data based on triplicate determinations in analytical runs on 3 different days. CV, Coefficient of variation; DFN, deviation from nominal concentration; LLOQ, lower limit of quantification; QC, quality control.

Table 4
Assay parameters for determination of analytes from tissue homogenate

Analyte	Concentration range (μM)	Retention time[a] (min)	LLOQ (μM)	Regression coefficient[b]
DNP-Cys	0.1–50	2.72 ± 0.004	0.1	0.9958–0.9972
DNP-SG	0.1–100	3.44 ± 0.012	0.1	0.9924–0.9999
DNP-NAc	0.25–20	6.70 ± 0.005	0.25	0.9986–0.9989
DNP	1.0–20	6.90 ± 0.051	1.0	0.9981–0.9998
CDNB	1.0–20	7.87 ± 0.035	1.0	0.9848–0.9998

LLOQ, Lower limit of quantification; sample volume = 100 μL.
[a] Mean ± S.D. for six replicates.
[b] Range.

interday results for accuracy and precision at the LLOQ and at the QC concentrations are presented in Table 5. For the QC samples, both interday and intraday accuracy showed less than 13.20% DFN and precision was within 9.37% CV, for all the analytes. There was a good linear relationship ($1/C$ weighted) between peak areas and $C(x)$ over the concentration range of 0.1–50 μM for DNP-Cys with linear regression yielding $y = (2146 \pm 7.82)x + (6.14 \pm 0.003)$, 0.1–100 μM for DNP-SG [$y = (2013 \pm 38.9)x + (19.34 \pm 0.18)$], 0.25–20 μM for DNP-NAc [$y = (4302 \pm 19.6)x + (23.65 \pm 0.01)$] and 1–20 μM for both DNP [$y = (581 \pm 89.2)x + (5.55 \pm 0.08)$] and CDNB [$y = (282.8 \pm 32.7)x + (6.62 \pm 0.09)$].

The relative recovery values based on a comparison of peak areas between spiked tissue homogenates and spiked homogenizing buffer samples at the QC concentrations for all the analytes are summarized in Table 5. Recovery values for all the analytes were greater than 80% and were acceptable for the purpose of placental drug transport studies. Recovery values appeared to decrease with increasing lipophilicity of the analytes and may reflect greater binding of the lipophilic compounds to placental tissue.

3.5. Stability

The stock solutions stored at −20 °C were stable for at least 6 months. The working solutions, standards and samples were maintained at 4 °C and were stable for at least 24 h. Short-term storage stability studies over a period of 2 weeks indicated no significant degradation for any of the analytes in PCA-treated buffer samples stored at −20 °C or PCA-treated tissue homogenate samples stored at −80 °C (Table 6).

3.6. Application

This method has been applied successfully to determine the concentration of DNP-SG and CDNB in buffer and tissue homogenate, without or with treatment with the ATPase inhibitor sodium orthovanadate and after brief exposure to 100 μM CDNB. Fig. 3 illustrates the time course of DNP-SG efflux from villous tissue fragments. DNP-SG efflux normalized to tissue DNP-SG content increased linearly with time up to 45 min and was significantly inhibited by 67.4 ± 6.53% ($p < 0.05$) upon treatment with ATPase inhibitor

Table 5
Assay validation results for determination of analytes from tissue homogenate

Sample	Nominal concentration (μM)	Intraday ($n = 6$)			Interday ($n = 9$)			Relative recovery[a] (%)
		Measured concentration[a] (μM)	Precision CV (%)	Accuracy DFN (%)	Measured concentration[a] (μM)	Precision CV (%)	Accuracy DFN (%)	
DNP-Cys								
LLOQ	0.1	0.089 ± 0.018	20.22	−11.04	0.091 ± 0.017	18.68	−9.17	–
QC1	0.3	0.329 ± 0.022	6.79	9.80	0.340 ± 0.028	8.13	13.20	106 ± 4.89
QC2	10	8.84 ± 0.35	3.98	−11.62	8.86 ± 0.28	3.15	−11.40	112 ± 3.32
QC3	40	40.2 ± 1.11	2.77	0.50	40.2 ± 0.98	2.48	0.46	108 ± 8.93
DNP-SG								
LLOQ	0.1	0.113 ± 0.011	9.66	12.81	0.088 ± 0.013	14.30	−11.92	–
QC1	0.25	0.222 ± 0.007	3.08	−11.03	0.226 ± 0.014	6.06	−9.41	97.7 ± 8.76
QC2	6	6.10 ± 0.04	0.67	1.73	6.01 ± 0.398	6.62	0.16	104 ± 6.05
QC3	80	81.0 ± 1.17	1.45	1.19	82.7 ± 1.21	1.47	3.37	113 ± 7.07
DNP-NAc								
LLOQ	0.25	0.261 ± 0.029	11.11	4.41	0.257 ± 0.031	12.06	2.80	–
QC1	0.6	0.669 ± 0.017	2.48	11.55	0.671 ± 0.020	2.91	11.90	93.2 ± 5.32
QC2	2	1.93 ± 0.02	1.16	−3.73	1.96 ± 0.04	1.90	−2.13	89.4 ± 6.31
QC3	16	16.3 ± 0.06	0.37	1.64	16.5 ± 0.24	1.44	3.04	91.7 ± 8.76
DNP								
LLOQ	1	0.922 ± 0.044	4.35	−8.12	1.03 ± 0.07	6.80	3.18	–
QC1	2	1.97 ± 0.03	1.56	−1.28	2.09 ± 0.19	9.07	4.54	86.9 ± 7.38
QC2	4	3.96 ± 0.03	0.79	−1.02	4.19 ± 0.39	9.37	4.66	87.5 ± 1.56
QC3	16	16.3 ± 0.28	1.72	1.68	16.7 ± 1.25	7.44	4.94	91.5 ± 6.73
CDNB								
LLOQ	1	0.912 ± 0.061	6.59	−9.01	0.87 ± 0.09	10.34	−13.00	–
QC1	2	2.13 ± 0.05	2.41	6.47	2.08 ± 0.13	6.10	3.90	82.6 ± 8.93
QC2	4	3.91 ± 0.09	2.32	−2.21	4.00 ± 0.13	3.23	0.07	81.8 ± 7.54
QC3	16	15.8 ± 0.42	2.68	−1.54	16.2 ± 0.51	3.14	1.21	83.7 ± 9.77

Intraday data based on triplicate determinations in two analytical runs on the same day. Interday data based on triplicate determinations in analytical runs on 3 different days. Relative recovery based on triplicate determinations in a single analytical run. CV, Coefficient of variation; DFN, deviation from nominal concentration; LLOQ, lower limit of quantification; QC, quality control.

[a] Mean ± S.D.

Table 6
Short-term storage stability in buffer and tissue homogenate

Sample	Nominal concentration (μM)	Buffer stability (%)	Tissue stability (%)
DNP-Cys			
QC1	0.3	102 ± 3.42	103 ± 2.97
QC2	10	98.7 ± 2.56	97.3 ± 5.12
QC3	40	99.6 ± 6.72	98.4 ± 4.31
DNP-SG			
QC1	0.25	97.3 ± 0.90	105 ± 4.10
QC2	6	102 ± 3.83	103 ± 0.59
QC3	80	97.8 ± 0.97	99.9 ± 1.52
DNP-NAc			
QC1	0.6	104 ± 2.89	98.6 ± 1.84
QC2	2	102 ± 7.83	99.2 ± 2.21
QC3	16	99.6 ± 4.22	98.8 ± 3.79
DNP			
QC1	2	104 ± 0.74	102 ± 7.49
QC2	4	104 ± 0.06	98.1 ± 11.12
QC3	16	95.8 ± 1.02	94.5 ± 0.81
CDNB			
QC1	2	98.3 ± 0.78	99.7 ± 1.62
QC2	4	99.3 ± 0.93	103 ± 2.17
QC3	16	97.5 ± 5.21	96.8 ± 1.93

Data (mean ± S.D.) represent analyte concentrations in 2 week stability samples as a percentage of corresponding analyte concentrations in freshly prepared QC samples.

sodium orthovanadate, indicating the involvement of ABC transporters in the apical efflux of DNP-SG from villous tissue fragments. Furthermore, the tissue DNP-SG content did not change upon sodium orthovanadate treatment, suggesting that this ATPase inhibitor does not affect GSTP1-1 activity required for DNP-SG formation. Upon exposure to 100 μM CDNB, the DNP-SG metabolite DNP-Cys was detected in the tissue homogenate and efflux buffer samples, but its amounts were very low (<10%) compared to DNP-SG content. DNP-NAc was not detected in any of the experimental samples. DNP and CDNB were detected in some of the samples, however the concentrations of these analytes were below the LLOQ for tissue exposed to 100 μM CDNB.

The paradigm of CDNB conjugation with glutathione and subsequent efflux of DNP-SG has been widely studied in several biological systems, but has not been studied in the human placenta. The coordinated function of glutathione-*S*-transferases and ABC transporters in the detoxification of CDNB has also been used as a mechanistic probe system to study the effects of pathophysiological conditions such as cholestasis [6,9,10], hyperbilirubinemia [17] and compromised liver function [16] on the enzyme and transporter functions in the rat liver. The assay reported in this paper facilitates extension of the concept of using CDNB as a probe substrate to study human placental drug disposition. CDNB disposition studies could be very useful to study human placental GSTP1-1 and ABC transporter activities, as well as the effect of pathophysiological conditions on placental drug disposition.

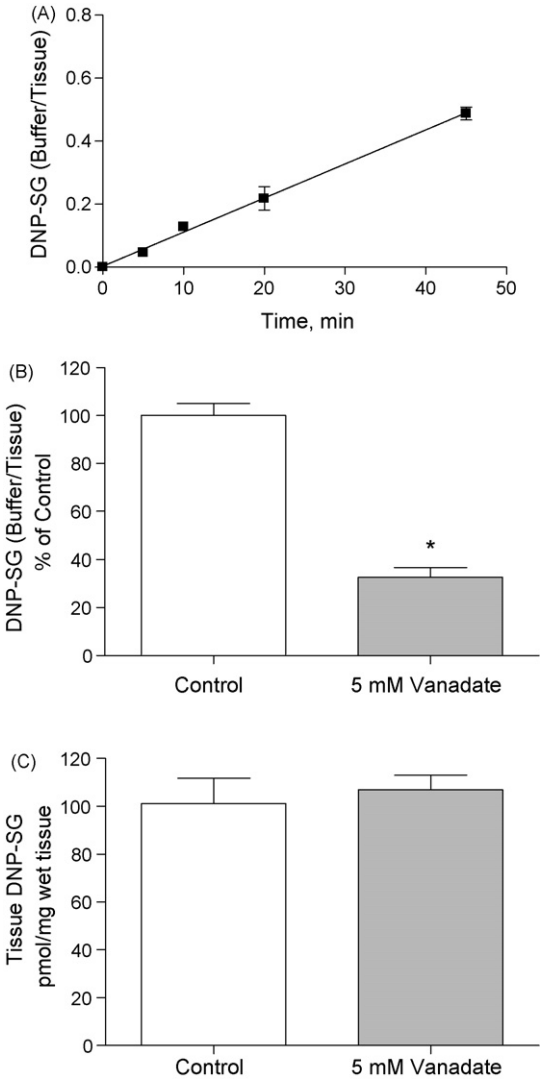

Fig. 3. DNP-SG amount in the buffer normalized to tissue DNP-SG after brief exposure to 100 μM CDNB for 5 min at 4 °C, followed by incubation with DPBS at 37 °C up to 45 min. Data represent mean ± S.D. from triplicate determinations in a representative placental tissue sample (A). DNP-SG amount in the buffer normalized to tissue DNP-SG (B) and tissue DNP-SG amounts (C) after 20 min incubation with DPBS at 37 °C in the presence or absence of sodium orthovanadate. Data represent mean ± S.E.M. from triplicate determinations in placental tissues from three patients. Asterisk (*) indicates $p < 0.05$ following a paired Student's *t*-test.

4. Conclusion

The present method is the first validated analytical method for the simultaneous detection of CDNB, DNP, DNP-SG, DNP-Cys and DNP-NAc from human placental tissue homogenate and DPBS. A simple pretreatment procedure, short run time, high sensitivity, good recovery and reproducibility are the salient features of this assay. Simultaneous measurement of CDNB, DNP-SG and it metabolites from human placental tissue homogenate and DPBS would provide a complete understanding of the CDNB detoxification mechanisms and would be helpful in establishing the functional role of ABC transporters such as ABCC1/2 and ABCG2 in the human placenta. The approach of combining 3 μm solid phase particle size, rapid gradient elu-

tion, and dual wavelength detection could be applied to analyze a wide range of drugs and metabolites. Such methods would be beneficial for studying metabolism and transport in the placenta and other tissues.

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