A SIMPLE COLORIMETRIC ASSAY FOR PHENOTYPING THE MAJOR HUMAN THERMOSTABLE PHENOL SULFOTRANSFERASE (SULT1A1) USING PLATELET CYTOSOLS

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ABSTRACT:

A thermostable phenol sulfotransferase, SULT1A1, has been implicated in numerous detoxification and bioactivation pathways; however, little is known regarding its endogenous function or its putative role in mediating risk for human environmental disease. A simple endpoint colorimetric assay is described that can be used for rapid phenotyping of SULT1A1 activity in human populations. The assay utilizes a microtiter-plate format and relatively small amounts of platelet cytosol-derived enzyme. The enzyme catalyzes the synthesis of 2-naphthylsulfate from 2-naphthol and 5'-phosphoadenosine 3'-phosphosulfate (PAPS), whereas addition of p-nitrophenyl sulfate to the assay contributes to an effective PAPS-regenerating system. In contrast to other sulfotransferase assay methods, 3'-phosphoadenosine 5'-phosphate (PAP) does not accumulate during the incubation to interfere with enzyme activity, but instead serves as a cofactor to cause the removal of sulfate from p-nitrophenyl sulfate to regenerate PAPS. This reaction concomitantly results in generation of p-nitrophenol that can be quantified colorimetrically at 405 nm (ε = 18,200 M⁻¹ cm⁻¹) to give an indirect measure of sulfotransferase activity. Using platelet enzyme preparations from adult human subjects, sulfation rates of two prototypical thermostable phenol sulfotransferase substrates (2-naphthol and p-nitrophenol) and one thermolabile phenol sulfotransferase substrate (dopamine) were determined using standard radiochemical protocols. These data were then compared with results from the colorimetric assay using 2-naphthol as substrate. There was a good correlation between the phenotyping assay and radiochemical assays for both 2-naphthol sulfotransferase and p-nitrophenol sulfotransferase activity (r = 0.85 and 0.69, respectively). However, SULT1A1 activity was approximately 10 to 20 times higher with the colorimetric determination. As anticipated, there was no correlation between SULT1A1 activity and dopamine sulfotransferase activity (r = 0.07) in these human platelet preparations. This inexpensive and rapid method for phenotyping SULT1A1 activity may help investigators assess a role for this enzyme in disease susceptibility.

Sulfation is a mechanism by which a wide variety of hormones, neurotransmitters, drugs, and xenobiotic compounds are detoxified (Jakoby and Zeigler, 1990). Alternatively, sulfation can be an important mechanism of bioactivation, because reactive sulfuric esters of hormones, neurotransmitters, and xenobiotics can bind covalently to DNA (Kadlubar et al., 1976; Kato and Yamazoe, 1987; Abu-Zeid et al., 1992). There are seven known human cytosolic enzymes involved in sulfation conjugation, an estrogen sulfotransferase (designated SULT1E1), three hydroxysteroid sulfotransferases (designated SULT2A1, SULT2B1a, and SULT2B1b), and three phenol sulfotransferases (PSTs), designated SULT1A1, SULT1A2, and SULT1A3 [reviewed in Her et al. (1998) and Sakakibara et al. (1998)]. Expression of other human cytosolic PSTs (SULT1C1, SULT1B1/2) have also been reported (Fujita et al., 1997; Sakakibara et al., 1998; Wang et al., 1998; Windmill et al., 1998). The thermolabile form of PST appears to be expressed in a variety of tissues, and is important for the conjugation of dopamine and other monoamines. The two most abundant PSTs, SULT1A1 and SULT1A2, detoxify numerous phenolic compounds, and are also thought to play the primary role in sulfotransferase-mediated activation of proximate carcinogens, such as N-hydroxy-2-acetylaminofluorene, N-hydroxy-4-aminobiphenyl, and N-hydroxy-2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine (Ozawa et al., 1994; Chou et al., 1995a,b). How-
ever, of these, only SULT1A1 is expressed at appreciable levels in human liver (Ozawa et al., 1998; named ST1A3 in this reference). Anderson et al. (1998) have further reported the purification of a single major thermostable PST, presumably SULT1A1, in human platelets. The thermolabile SULT1A3, as measured by dopamine sulfotransferase activity, is also expressed in significant amounts in human platelets (Anderson et al., 1998). However, on a per milligram of protein basis, both SULT1A1 and SULT1A3 are expressed at relatively lower levels in platelets as compared with human liver. Other sulfotransferase isoenzymes have not been reported in human platelets.

Disease mechanisms related to SULT1A1 and SULT1A3 expression have been studied previously using human peripheral blood. Weinshilboun and collaborators reported good concordance between thermolabile PST activity in human platelets and jejunal mucosa, liver, and cerebral cortex; however, there was no such correlation for the thermolabile PST in platelets and these tissues (Weinshilboun, 1988; Sundaram et al., 1989; Weinshilboun, 1990). Thus, the coordinate regulation between SULT1A1 in both liver and blood platelets provides the opportunity for the utilization of minimally invasive phenotyping methods for estimating hepatic SULT1A1 levels and hence its role in carcinogenesis and drug metabolism.

Pharmacogenetic studies of platelet-derived SULT1A1 and SULT1A3 activities further indicate that the activities of these two isoenzymes are regulated by separate genetic mechanisms or polymorphisms (Price et al., 1988; Raffogianis et al., 1996). With respect to SULT1A1 activity, this may account, at least in part, for up to a 50-fold individual variability in phenotype (Raffogianis et al., 1997). A largely genetic basis for variability in human SULT1A1 activity is also supported by the recent characterization of multiple variant alleles (Henkel et al., 1995; Weinshilboun et al., 1997; Ozawa et al., 1998). Less is known about host-specific and environmental factors that impact on sulfotransferase expression and possibly contribute to disease susceptibility. However, numerous studies in animal, human, and cell models suggest modulation by gender, hormones, disease, developmental factors, hypoxia, diet, season, and xenobioc exposure (reviewed by Coughtrie et al., 1998).

At present, the link between SULT1A1 activity and risk for human disease is relatively unexplored. The standard phenotyping assay involves the use of expensive radioactive substrates and labor-intensive precipitation and centrifugation steps, factors that limit its application to large scale phenotyping studies. However, in this report, a simple and reproducible endpoint colorimetric assay is described that has been adapted to a 96-well microtiter plate format. The assay was validated by measuring 2-naphthol sulfation rates in preparations of normal human platelets (n = 8), by correlating these results to those obtained using standard radiochemical methods, and by product identification using liquid chromatography-mass spectrometry. Three prototypical SULT substrates have been used to verify that the colorimetric assay is specific for SULT1A1, and not for SULT1A2 or SULT1A3, and were supported by measurements of either SULT mRNA or immunoreactive protein and by the use of specific inhibitors. These validation studies are critical for showing that the colorimetric assay is a useful and inexpensive alternative, particularly for SULT1A1 phenotyping of population-based molecular epidemiological studies.

Materials and Methods

Reagents. The following chemicals (and their sources) were obtained commercially: phosphate-buffered saline (pH 7.4; #1000-3), trisodium citrate, citric acid, triethanolamine, 2-mercaptoethanol, acetylsalicyclic acid, 2-naphthol, potassium monobasic and dibasic phosphate, magnesium chloride, 5’-phosphoadenosine 3’-phosphosulfate (PAPS), p-nitrophenylsulfate, pargyline, dopamine, barium hydroxide, zinc chloride, and barium acetate from the Sigma Chemical Co. (St. Louis, MO); N,N-dimethylformamide, triethylamine, and 1,3-dicyclohexylcarbodiimide from Aldrich Chemical Co., Inc. (Milwaukee, WI); dextrose from Fisher Scientific (Houston, TX); sucrose from Bio-Rad Laboratories (Hercules, CA); and 135SI-PAPS from New England Nuclear, Inc. (Boston, MA). Rabbit anti-human SULT1B1/2 (Wang et al., 1998) was kindly provided by Dr. Charles Falany (University of Alabama at Birmingham).

The citrate/phosphate-buffered saline was prepared by constituting a 15:85 (v/v) mixture of 1) 20 mM sodium citrate buffer, pH 7.4, containing 20 mM dextrose with 2) phosphate-buffered saline (pH 7.4), 2-Naphthylsulfate was prepared as described in Ozawa et al. (1998), with the condensation with sulfuric acid using dicyclohexylcarbodiimide. Briefly, 28.8 mg of 2-naphthol was dissolved in 10 ml of dimethylformamide, 13 μl of concentrated H2SO4 was added, and this was followed by 226 mg of dicyclohexylcarbodiimide. The insoluble dicyclohexylurea was removed by centrifugation; triethylamine (45 μl) was added to the supernatant to form the triethylamine salt and the 2-naphthylsulfate product was crystallized by cooling the solution on ice. The yield was 40%, and the product was judged to be >99% pure by liquid chromatography-mass spectrometry [vide infra; (M+H+) = 225].

Human Subjects and Blood Processing. To assess intrasubject variability in platelet PST activities, blood platelets were isolated (on four separate occasions at weekly intervals) from healthy human volunteers, 24 to 65 years old, who were participants in an ongoing colon cancer case control study. For each individual, approximately 24 to 32 ml of blood was collected in four to six Vacutainer tubes (“yellow-top ACD” tubes; Becton-Dickinson, from Fisher Scientific, Houston, TX). The tubes were maintained at room temperature with gentle rocking for up to 24 h before processing. Isolation of the platelets from blood cells was then carried out by differential centrifugation, also at room temperature. Briefly, blood lymphocytes and platelets were collected from the upper interphase of discontinuous Histopaque gradients (polysucrose; Sigma) that were prepared in four to six (15 ml) conical polystyrene centrifuge tubes according to the manufacturer’s instructions.

Three citrate/phosphate-buffered saline washes, interspersed with 150 mg centrifugation steps, removed contaminating lymphocytes from platelets, which were retained in the supernatants. The washes were pooled in 50-ml polystyrene centrifuge tubes, and the platelets were sedimented by centrifugation at 500g. The purified platelet pellets were then resuspended in buffer (10 mM triethanolamine-HCl buffer, 0.25 M sucrose, 5 mM 2-mercaptoethanol, and 67 μM acetylsalicylic acid, pH 7.4) and transferred to standard microcentrifuge tubes. A small aliquot was set aside for determination of platelet yield and purity (Model STKS; Cytometer Corp., Irving, TX). By this method, isolated platelets routinely showed negligible contamination with other cell types (<0.08%). Platelets used in correlation studies were obtained from these and additional control subjects participating in an ongoing colon cancer case control study.

Human livers were obtained as surgical samples from the John L. McClellan Memorial Veterans’ Hospital and the U.S. Cooperative Tissue Network. These tissues were excess surgical samples that were immediately frozen in liquid N2 and stored at −80°C before use. Cytosols were prepared as described by Chou et al. (1995a,b).

Immunoblotting for SULT1B2 was carried out by the method of Towbin et al. (1979) using 1:1000-fold dilution of the anti-SULT1B1/2. Detection of the immunoreactive bands was performed using the ECL chemiluminescence method from Amersham Pharmacia Biotech (Arlington Heights, IL).

Determination of SULT1A1, SULT1A2, and SULT1A3 mRNA transcripts in human platelets was performed according to the method of Ozawa et al. (1998). Total RNA was isolated from platelets by the acid guanidinium isothiocyanate method using TriReagent (Molecular Research Center, Cincinnati, OH) according to the manufacturer’s instructions. RNA (10 μg) was reverse-transcribed using the Promega (Madison, WI) Access reverse transcription-polymerase chain reaction system, which uses avian myeloblastosis virus reverse transcriptase for first strand cDNA synthesis. Second strand synthesis and subsequent DNA amplification were catalyzed by the thermostable T7 DNA polymerase from Thermus flavus using the specific primers described in Ozawa et al. (1998).

Preparation of Platelet Samples for PST Assays. After addition of a
sonication solution (0.1 ml/ml) containing 1 M KCl, 10 mM EDTA, and 0.3% 2-mercaptoethanol, purified platelet suspensions were subjected to three 3-s “bursts” of a sonicator, while on ice. Supernatants were recovered after a 10-min spin in a refrigerated microcentrifuge (14,000g, 4°C), removing intact cells and most subcellular membrane contaminants that contribute to optical interference. In some experiments, a further ultracentrifugation step (100,000g for 1 h, 4°C) replaced the microcentrifuge spin. If time permits, this step is recommended, because it minimizes potential optical interference in some samples. For either platelet supernatant isolation procedure, several aliquots were prepared and frozen at −80°C for up to 3 months without any detectable effects on enzyme activities. Samples were subjected to a single freeze-thaw cycle before assay.

Comparison of SULT1A1 Activity by Colorimetric and Radiochemical Assays. In the colorimetric assay, SULT1A1 activity was measured as the release of p-nitrophenol from a PAPS-regenerating system originally developed by Mulder et al. (1977). The basis for the SULT1A1 phenotyping assay is depicted in Fig. 1A. Although some overlapping substrate specificity has been reported for various sulfotransferase isoforms, reported $K_m$ values are approximately two orders of magnitude lower for reactions with prototypical substrates compared with nonpreferred substrates (Veronese et al., 1994). For the phenotyping assays reported here, the prototypical SULT1A1 substrate, 2-naphthol, was used.

For assays using liver cytosol (0.1–0.5 mg/ml), the reaction mixtures (1 ml) contained 50 mM potassium phosphate buffer (pH 6.5), 5 mM magnesium chloride, 20 μM PAPS, 5 mM p-nitrophosphatase, and 0.01 to 0.10 mM substrate. To overcome problems associated with relatively low PAPS activity in platelet supernatants (cf. liver) and with limited sample sizes, a 96-well microtiter plate format was developed. These reaction mixtures (100 μl) contained 35 to 70 μl of the 14,000g supernatant (approximately 20–40 μg of protein), 50 mM potassium phosphate buffer (pH 6.5), 5 mM MgCl2, 20 μM PAPS, 5 mM p-nitrophosphatase, and 0.1 mM 2-naphthol. Triplicate samples were prepared with and without substrate. After incubation in a humidified 37°C incubator for 45 min, the reaction was terminated by the addition of barium hydroxide, zinc chloride, and barium acetate to precipitate excess $[^{35}S]$PAPS. After centrifugation, each sample was subjected to a second centrifugation, and the individual supernatants were finally transferred to vials, where the radioactivity was measured by liquid scintillation counting. The difference in radioactivity due to addition of substrate was then determined. The results were expressed as average reaction rates (pmol/min/mg of protein) from triplicate determinations, ± S.D.

Results
Substrate Selection and Assay Linearity. Initial studies were carried out with liver cytosol using a variety of phenolic substrates at a range of concentrations. Of these, 2-naphthol exhibited the highest turnover (Fig. 2). A 2-naphthol substrate concentration of 0.1 mM was optimal for the colorimetric assay, as higher concentrations resulted in significant substrate inhibition, a characteristic not uncommon for this and other sulfotransferase isoforms (Campbell et al., 1987). Although the colorimetric assay was linear with time for 15 min (Fig. 2) and proportional to protein concentration up to 0.5 mg/ml using the liver 100,000g supernatant with the microtiter plate format, the reaction was found to be proportional to platelet protein concentration and time with linear for at least 45 min using either the 14,000g (up to 0.2 mg/ml protein) or the 100,000g (up to 0.04 mg/ml protein) platelet supernatant preparations. Protein values greater than this resulted in p-nitrophenol absorbance values (>0.400) that did not obey Beer’s law. Moreover, studies with recombinant SULT1A1 and SULT1A2 (Ozawa et al., 1994) showed that the former had 20-fold higher activity toward 2-naphthol, thus supporting our selection of this substrate for SULT1A1 phenotyping. Moreover, quercetin (10 μM) and 2,6-dichloro-4-nitrophenol (10 μM), which are reported to be selective for inhibition of SULT1A1, and not SULT1A2 or other SULTs (Glatt et al., 1999; Raftogianis et al., 1999), inhibited the 2-naphthol colorimetric assay by 60% and 90%, respectively. Furthermore, the addition of substrates (100 mM) for SULT1A3 (dopamine), SULT1B1/2 (triiodothyronine), and SULT1E1 (estrone) did not contribute to absorbance at 405 nm in this phenotyping assay. Finally, an antibody to human SULT1B2 did show cross-reactivity toward platelet cytosol (Fig. 3A). Similarly, measurement of SULT1A1, -1A2, and -1A3 mRNA transcripts in platelet RNA revealed the presence of only SULT1A1 and -1A3 mRNAs (SULT1A3 does not contribute to the assay); SULT1A2 mRNA was not detected (Fig. 3B). Thus, and protein were used in each assay set; time-dependent changes in optical density in these reactions were not detectable. Protein was measured using a microscale method (Bio-Rad Coomassie dye reagent) with bovine serum albumin as the reference standard.

This assay was validated by liquid chromatography-mass spectrometry on a Micromass Platform II single quadrupole mass spectrometer (Manchester, UK), equipped with an electrospray interface with an ion source temperature of 150°C. Separation was achieved on a Beta Basic C18 column (2 × 150 mm; 3 μm), with isocratic elution (40% acetonitrile in water); the flow rate was 0.2 ml/min. In a typical experiment, the colorimetric assay yielded a measure of 23.6 pmol of 2-naphthylsulfate, which was determined by liquid chromatography-mass spectrometry to be 24.2 pmol. For the radiochemical SULT assays, measurement techniques were adapted from the method of Foldes and Meek (1973). The basis for the radiochemical SULT1A1 assay is depicted in Fig. 1B. Triplicate assay tubes were prepared (plus and minus substrate). Briefly, reaction mixtures for 2-naphthol sulfation consisted of 50 mM potassium phosphate buffer (pH 6.5), 5 mM magnesium chloride, 20 μM $[^{35}S]$PAPS, and platelet supernatant, in the presence or absence of 2-naphthol (0.1 mM). For p-nitrophenol assays, the reaction mixture consisted of 50 mM potassium phosphate buffer (pH 6.5), 5 mM MgCl2, 20 μM $[^{35}S]$PAPS, platelet supernatant, in the presence or absence of p-nitrophenol (4 μM). For dopamine assays, the reaction mixture contained 50 mM potassium phosphate (pH 6.8), 5 mM MgCl2, 20 μM $[^{35}S]$PAPS, 1 μM pargyline (monoamine oxidase inhibitor), platelet supernatant, plus or minus dopamine (10 μM). These mixtures were incubated for 10 min at 37°C and then were terminated by the addition of barium hydroxide, zinc chloride, and barium acetate to precipitate excess $[^{35}S]$PAPS. After centrifugation, each sample was subjected to a second centrifugation, and the individual supernatants were finally transferred to vials, where the radioactivity was measured using a Micromass Platform II single quadrupole mass spectrometer (Manchester, UK) and a Microscale method (Bio-Rad Coomassie dye reagent) with bovine serum albumin as the reference standard. Protein was measured using a microscale method (Bio-Rad Coomassie dye reagent) with bovine serum albumin as the reference standard.

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SULT1A1 is the only isoform contributing to this assay in platelet cytosol.

**Substrate Specificity.** To validate the use of this phenotype (colorimetric) assay for SULT1A1 activity in platelet fractions, a series of comparisons were made to assess the correlation with standard radiochemical PST assays. As shown in Fig. 4A, a high degree of correlation ($r = 0.87$) was found between 2-naphthol PST activities measured colorimetrically and by radiochemical detection ($n = 8$). Note that actual values were much higher in the colorimetric assay (0.2–3.7 nmol/min/mg) than in the corresponding radiochemical assay (15–170 pmol/min/mg) for the same samples.

The correlation was also good ($r = 0.69$) between the platelet phenotyping assay and $p$-nitrophenol PST measured radiochemically (Fig. 4B), and this correlation was identical with that for 2-naphthol PST versus $p$-nitrophenol PST, both measured radiochemically (Fig. 4C). As anticipated, the lowest correlations were found for comparsons of 2-naphthol and dopamine sulfotransferase activities. Whether 2-naphthol ST activity was measured colorimetrically or radiochemically (Fig. 5, A and B, respectively), the correlation with dopamine ST activity was very poor. The correlation coefficient was 0.07 when 2-naphthol PST was measured by the colorimetric assay and was 0.16 when 2-naphthol PST was measured by the standard radiochemical assay. This confirmed that the colorimetric assay selectively measures SULT1A1 activity and not SULT1A2 or SULT1A3 activity.

**Intraindividual Variability.** Figure 6 shows the relatively low intraindividual variability when healthy human adult volunteers were phenotyped for SULT1A1 on four occasions, with sampling at weekly intervals. Individuals that had the highest sulfotransferase activities on the initial assay also ranked highest on subsequent assays. Likewise, low 2-naphthol sulfotransferase activity was a good correlate of the slow SULT1A1 phenotype, whether based on single or multiple samples.

**Discussion**

A high correlation between 2-naphthol sulfotransferase activities measured by the colorimetric and radiochemical PST methods was observed, indicating that both assays measure the same sulfotransferase isofrom. Irrespective of the assay technique, there was a good correlation between 2-naphthol and $p$-nitrophenol sulfotransferase activities. This was expected, because both 2-naphthol and $p$-nitrophenol are considered prototypical substrates for the SULT1A1 isoform. The low specific activity of the SULT1A2 isoform toward 2-naphthol and the results of enzyme purification studies in human platelets (Anderson et al., 1998) essentially rule out any contribution of SULT1A2 in platelet supernatants to the SULT1A1 phenotyping assay. Similarly, SULT1A3 activities using dopamine as a substrate showed no correlation with the 2-naphthol phenotyping assay, thus excluding this isofrom as a confounding factor.

Activity values derived from the colorimetric 2-naphthol assay were consistently higher than the corresponding radiochemical assay. One possible explanation may be that $3'$-phosphoadenosine $5'$-phosphate (PAP) is generated as a reaction byproduct in the traditional radiochemically based assays but not in the colorimetric assay (cf. Fig. 1, A and B). PAP is a known potent inhibitor of sulfotransferase enzymes (Rens-Domiano and Roth, 1987) but is not accumulated in the colorimetric assay. Therefore, compared with other standard assay techniques, the colorimetric method may give the better estimate of SULT1A1 reaction rates.

Within our small sample size, interindividual variability in SULT1A1 activities ranged from 1 to 2.4 nmol/min/mg ($n = 8$). Other reports show 50-fold or greater variability (Frame et al., 1997; Rafto-
gianis et al., 1997). Intraindividual and intra-assay variabilities were very low with few identifiable technical problems for effective phenotyping. As with other experimental approaches, these results support the idea that SULT1A1 expression has a strong genetic component. It is interesting that certain sulfotransferase isoforms in humans and animals are modulated by season (Marazziti et al., 1995), hormones (Runge-Morris, 1998), disease state (el Mouelhi et al., 1987), diet, and other factors (Burchell and Coughtrie, 1997). To date, nongenetic factors that modulate SULT1A1 activity have not been reported. However, using this simple phenotyping assay, nongenetic as well as genetic factors may be explored readily. For example, the assay may be useful for screening variant SULT1A1 allele(s) to correlate with SULT1A1 activity and risk for human disease. It may also be useful for characterization of age- and disease-associated alterations of this xenobiotic-metabolizing enzyme.

The assay described in this report is inexpensive and simple to perform. The microtiter format greatly decreases the assay time per sample, such that in a few days to weeks, several hundred cytosol samples may be measured for SULT1A1 activity. For example, using this phenotyping technique, we were able to complete phenotyping of 367 individual blood samples in 10 working days (Frame et al., 1997).

Statistical analyses were conducted using the Spearman rank order correlation test. Platelet cytosols from the same eight donors were used for all the assays shown in Figs. 2 and 3. ST, sulfotransferase activity.

Fig. 4. Correlation plots between the colorimetric platelet phenotyping assay and the radiochemical assay for 2-naphthol (A) and p-nitrophenol (B) and between 2-naphthol and p-nitrophenol using the radiochemical assay (C).

Fig. 5. Correlation plots between the colorimetric phenotyping assay (A) and the radiochemical assay (B) using 2-naphthol and dopamine as substrates.
anticipated that this simple phenotyping method for SULT1A1 will improve the investigation of genetic, environmental, and host-specific factors that impact on SULT1A1 expression and disease risk.

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References


