Development of a Semi-Quantitative Assay to Detect Full-Length CYP2C19 RNA

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ABSTRACT

Cytochrome P450 2C19 (CYP2C19) is the enzyme responsible for the metabolism of a number of drugs, including anticonvulsants and antidepressants. We have developed a semi-quantitative competitive RT-PCR assay to estimate the degree of expression of the full-length CYP2C19 message. This assay used a known quantity of internally deleted CYP2C19 RNA to quantitate the RT-PCR products of the CYP2C19 transcript in the RNA extracted from tissues. We determined that this method is sensitive and reproducible in assaying for CYP2C19 RNA in human livers. The lowest detectable amount of competitor RNA was 0.166 fg or 270 copies of CYP2C19 competitor RNA. Using human liver samples containing $3-23 \times 10^5$ copies of CYP2C19 RNA, we found the assay to be reproducible with a coefficient of intra- and interday variation of 11% and 20%, respectively. Using this assay, we measured full-length CYP2C19 RNA in 10 human livers. We found the CYP2C19 transcripts range from $0.1-23 \times 10^5$ copies/mg liver total RNA. The analysis of CYP2C19 transcripts for liver of *1 and *2 genotypes, based on restriction enzyme digest analysis of RT-PCR products, suggests that only normal (*1), not the variant (*2) copy of full-length CYP2C19 RNA, was detectable in these livers. We report for the first time the quantification of full-length CYP2C19 RNA in livers.

INTRODUCTION

The cytochrome P450 (CYP) enzymes are a super family of heme-containing enzymes primarily responsible for the oxidative metabolism of drugs, environmental contaminants and xenobiotics (16). The CYP2C19 isoenzyme is responsible for the metabolism of a number of drugs including S-mephenytoin (15,38), diazepam (6,32), imipramine (31), propranolol (35), hydantoins and certain barbiturates (19,38), omeprazole (3), chloroguanide (36), citalopram (30) and clomipramine (22). In vivo, CYP2C19 activity of individuals can be phenotyped according to their ability to hydroxylate the S-enantiomer of the anticonvulsant drug mephenytoin (at the 4' position). Additional studies suggest that CYP2C19 protein content correlates with S-mephenytoin-4'-hydroxylase activities of human liver microsomes (15,39).

In addition, two single-point mutations originally described by de Moraes et al. (11,12) for this enzyme (CYP2C19*2 and CYP2C19*3 by the new nomenclature) (9) have been shown to significantly reduce CYP2C19 enzyme activity. These two genetic defects account for greater than 99% of Oriental and about 87% of Caucasian poor metabolizers of CYP2C19 substrates. Recently, three additional mutations, CYP2C19*4 (13), CYP2C19*5 (17,40) and CYP2C19*6 (18), were described to have lower frequencies.

To further elucidate the mechanisms of genetic inheritance and regulation of polymorphic enzymes at all levels (including transcriptional and translation-

al), many studies have been undertaken that have only qualitatively described the detection of small RNA fragments specific for CYP2C19. However, detection of small fragments (usually greater than 500 bases in length) (11) may not provide an estimate of the amount of full-length CYP2C19 RNA transcript that is essential for synthesis of functional CYP2C19 protein. Therefore, we have developed a competitive RT-PCR assay to estimate full-length CYP2C19 RNA in human livers.

MATERIALS AND METHODS

Human Liver Samples

All the human livers were obtained from the University of Washington Solid Organ Transplant Program. Livers were harvested from brain-dead donors and were rejected for transplantation for a variety of reasons, the most common being a high degree of fatty infiltration. Harvested livers were perfused with cold storage solution and kept on ice until they were dissected into small pieces. The tissue pieces were then stored at -80°C for long-term preservation. All procedures were carried out with the approval of the University of Washington Institutional Human Subjects Review Board.

Total RNA Isolation

Approximately 10 mg tissue from each liver were crushed and homogenized in liquid nitrogen. Total RNA was extracted using Purescript® RNA

isolation kit (GENTRA Systems, Minneapolis, MN, USA) according to the manufacturer's instruction. The RNA concentration was determined by measuring RNA fluorescence in ethidium bromide with a spectrofluorometer (Hitachi F4500) set at $\lambda_{\text{ex}} = 287 \text{ nm}$, $\lambda_{\text{em}} = 618 \text{ nm}$, using yeast RNA as a standard. The RNA quality was verified to contain similar amounts of β -actin RNA/ng total RNA by RT-PCR assay as described (41).

Design and Synthesis of Internal Control RNA Competitor

DNA construct. The competitor RNA was created by deleting a 669-bp fragment from CYP2C19 cDNA plasmid (4.7 kb in length) (kindly provided by Dr. J.A. Goldstein, National Institute of Environmental Health Science, Research Triangle Park, NC, USA) (28) using *StyI*; the digested products were separated on a 1% agarose gel. The deleted CYP2C19 plasmid DNA fragments were extracted from the gel using QIAquick™ gel extraction kit (Qiagen, Valencia, CA, USA) and ligated using T4 DNA ligase (Life Technologies, Rockville, MD, USA). To facilitate the production of competitor RNA, the ligated CYP2C19 plasmid was transformed into DH5 α supercompetent cells. The modified 2C19 plasmid DNA was extracted from an overnight culture using a QIAprep spin miniprep kit (Qiagen). Because the CYP2C19 plasmid (pBluescript® SK ($\pm$) phagemid) contained a T3/7 promoter, required for the in vitro RNA transcription protocol, the plasmid DNA was used to synthesize the RNA transcript without further manipulation.

RNA transcripts. To obtain CYP2C19 transcripts for the internal competitor control, the modified CYP2C19 plasmid DNA was first linearized with *EcoRV*. After removal of protein with proteinase K (Life Technologies), the DNA template was purified by phenol-chloroform-isoamyl alcohol (25:24:1) extraction and ethanol precipitation. The DNA pellet was dissolved in DNase-RNase-free water (Sigma, St. Louis, MO, USA); this plasmid DNA solution was used as a template for transcription. The transcription reaction was performed using

a RNA transcription kit according to the manufacturer's instructions (Stratagene, La Jolla, CA, USA). The transcription mixture [1 $\times$ transcription buffer, 1 μg template, 1 μL 10 mM rNTP (rATP, rGTP, rCTP and rUTP), 1 μL 0.75 M DTT, 10 U T3 RNA polymerase and RNase-free water to a total volume of 25 μL] was incubated at 37°C for 30 min. The transcription products were treated with RNase-free DNase I (Life Technologies) at 37°C for 15 min to remove the DNA template and extracted twice with an equal volume of phenol-chloroform-isoamyl alcohol. The upper RNA-containing phase was transferred to a fresh tube and precipitated with ethanol and sodium acetate (pH 5.2); the RNA pellet was washed with 70% ethanol and suspended in RNase-free water. The deleted CYP2C19 internal control RNA product was 811 bases in length. A positive CYP2C19 RNA strand, 1480 bases long and referred to as the wild-type (wt) (*1) positive control standard, was synthesized in the same way as the internal control RNA. The integrity of both RNA strands was analyzed on a 1% agarose gel. Both were quantified by measuring the absorbance at 260 nm and stored at -80°C.

Characterization and Titration of Full-Length and Internally Deleted CYP2C19 RNA

To confirm that the positive RNA and internal control RNA were the correct products, both samples were subjected to RT-PCR. The RT-PCR product of CYP2C19 RNA was digested with *EcoRI*. RT-PCR products and digested products were subjected to electrophoresis on a 1% agarose gel and stained with ethidium bromide. Because the *EcoRI* restriction site was deleted in the internal control RNA, cleavage of the *1 standard RNA produced only two fragments, 841 bp and 639 bp in length. When the purified CYP2C19 RNA was subjected to PCR in the absence of reverse transcriptase, no CYP2C19 product was detected, indicating that the RNA preparations were free of DNA contamination. The purified positive and internal control RNA preparations were used for all subsequent experiments.

Northern Blot Analysis

Northern blot analysis of CYP2C19 RNA was performed by separating various amounts of CYP2C19 RNA transcripts on a 1% agarose gel containing formaldehyde as previously described (29). After transfer to a nitrocellulose membrane, the membrane was hybridized with the 285-bp cDNA probe (561–846 bp of CYP2C19 cDNA sequence), biotin-labeled according to the BioNick labeling system (biotin-14-dATP) and developed using the AP-streptavidin PhotoGene nucleic acid detection system (both from Life Technologies).

RT-PCR Condition

One-step RT-PCR assays using Titan™ one-tube RT-PCR system (Roche Molecular Biochemicals, Mannheim, Germany) were performed in a final volume of 50 μL . A typical reaction uses two mixtures, with all reagents added in the following order. Mixture 1 consisted of 0.2 mM each dNTP (dATP, dCTP, dGTP and dTTP; Promega, Madison, WI, USA), 0.4 μM each primer (Life Technologies), 5 mM DTT, 10 U RNase inhibitor (Promega), 5–500 ng total liver RNA; 1.6 fg internal control RNA was added when quantitating products, and RNase-free water was added to a final volume 25 μL , all in a nuclease-free microcentrifuge tube that was kept on ice. Mixture 2 consisted of 10 μL 5 $\times$ RT-PCR buffer, 2 mM MgCl_2 , 1 μL enzyme mixture (AMV and Expand™ High Fidelity PCR System; Roche Molecular Biochemicals, Mannheim, Germany), and RNase-free water was added the same way as for Mixture 1. Primers were designed based on CYP2C19 sequences available in the GenBank® database (accession no. M61854) with the assistance of a primer design software program.

For full-length CYP2C19 RT-PCR, the primers sequences were as follows. Forward primer (6–26): 5'-ATGGATCCTTTGTGGTCCTT-3'. Reverse primer (1485–1466): 5'-TGCTTCTTCA-GACAGGAATG-3'.

The primers for the short CYP2C19 RNA RT-PCR assay were the same as that described by de Morais et al. (11).

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Forward primer: 5'-ATTGAATGAAA-ACATCAGGATTG-3'. Reverse primer: 5'-GTAAGTCAGCTGCAGTGA-TTA-3'.

Twenty-five microliters each of mixture 1 and 2 were mixed into a 0.2-mL thin-walled PCR tube with attached dome cap (Scientific, Ocala, FL, USA). The reaction was performed in a GeneAmp® PCR system, model 9600 thermocycler (PE Biosystems, Foster City, CA, USA). Reverse transcription was performed at 50°C for 30 min. PCR amplification started with an initial denaturation at 94°C for 2 min, followed by 10 cycles of denaturation at 94°C for 30 s, annealing at 56°C for 30 s and extension at 68°C for 1.5 min. This was followed by another 25 cycles of denaturation at 94°C for 30 s, annealing at 60°C for 30 s and extension at 68°C for 1.5 min, adding 5 s to each cycle for elongation. The final extension step was held at 68°C for 7 min. Six microliters of RT-PCR products were separated on a 1% agarose gel and stained with ethidium bromide for fluorography. The liver samples and CYP2C19 standard amplification products (1480 bp) and the competitor products (811 bp) were well separated under these conditions.

Measurement of CYP2C19 mRNA of Liver Samples

Five hundred nanograms of total liver RNA were used as template to perform RT-PCR. The RT-PCR products were subjected to electrophoresis on a 1% agarose gel stained with ethidium bromide. According to the intensity of the band, we estimated and made serial (three- to fivefold) dilutions of total RNA and fixing competitor RNA (1.7 fg). The total RNA of samples was subsequently titrated. The concentration of CYP2C19 RNA in liver tissue (copies/ μ g total RNA) was calculated by an analysis of the digital image captured with a Kodak® DC120 camera (Eastman Kodak, Rochester, NY, USA) using the method described by Rasband et al. CYP2C19 RNA copy estimates were obtained from the average of duplicate RT-PCR assays for each test sample after accounting for the differences in lengths of the product and the competitor (i.e., 1.4 vs. 0.8 kb), similar to estimates described by Piatak et al (25).

Genotyping Procedure

Detection of the normal (CYP2C19*1) and defective (CYP2C19*2 and CYP2C19*3) alleles was performed by PCR-restriction enzyme analysis using the method described by de Morais et al. (11,12).

CYP2C19 Enzyme Activity

The enzyme activity of CYP2C19 was quantitated with microsomes isolated from respective liver samples. The hydroxylase activity of *S*-mephenytoin was determined according to Bajpai et al. (4) with an HPLC assay after incubating the liver microsome in the presence of 50 μ M substrate. Data were expressed as pmol of hydroxylated product formation/min/mg microsomal protein.

RESULTS

Characterization of Internally Controlled RNA Assay for CYP2C19 RNA

We first used the CYP2C19 and internally deleted CYP2C19 internal competitor control RNA to optimize the assay conditions. The design of internal control with the 669-bp internal deletion of CYP2C19 RNA permitted the use of one set of primers and produced both the 811-bp and 1480-bp CYP2C19 products as detected on agarose gel, well separated from each other (Figure 1). Typical analyses of CYP2C19 RNA are shown in Figure 1. The amount of CYP2C19 RNA is estimated based on the dilution in which the intensity of the CYP2C19 product and the internal control bands are equivalent after considering the differences in base pair length. After several rounds of optimization, we found that a minimum of 0.166 fg competitor or internal control RNA was required to produce a consistent, clearly detectable product. This amount is equivalent to 270 copies of RNA (Figure 1, panels A and B, lane 6). In contrast, at least 2.1×10^8 copies RNA or 2.2 ng RNA transcript is needed to be detectable in Northern blot analysis (Figure 2). For all the experiments described below, we used 2700 copies of internal control

RNA to ensure consistency and reproducibility in detection of CYP2C19 in RNA isolated from liver.

Reproducibility of the Full-Length CYP2C19 RNA Assay

To determine the variation of the assay, we selected four human liver samples and performed the assay beginning with RNA isolation to determine CYP2C19 RNA levels. We used the optimized, internally controlled RT-PCR by repeated testing at the same time (intraday) and on different days (interday). The results are summarized in Table 1. Using the liver samples containing $3\text{--}23 \times 10^5$ copies CYP2C19 RNA, we found that the assay was reproducible with coefficients of variation for intra- and interday reproducibility of 11% and 20%, respectively.

Figure 1. Representative gel analysis of CYP2C19 quantitative competitive RT-PCR assay product. CYP2C19 RNA and control (competitor) RNA were mixed and assayed in the same reaction as described in Materials and Methods. The products, 1.5 and 0.8 kb, respectively, were visualized after separating them on an agarose gel and staining with 1% ethidium bromide. Panel A: RT-PCR product of varying levels of CYP2C19 RNA with constant level of competitor RNA. Lane 1, 1-kb DNA ladder; lane 2, negative control, which included all reagents except RNA template; lane 3, 270 copies of competitor RNA were added without CYP2C19 RNA; lanes 4–8, 270 copies of competitor RNA plus fourfold serial dilutions (4.5, 1.1, 0.28, 0.07 and 0.02 fg) of CYP2C19 RNA. Panel B: RT-PCR product of varying competitor RNA with constant amount (0.28 fg) CYP2C19 RNA. Lane 1, 1-kb DNA ladder; lane 2, negative control, which included all reagents except RNA template; lane 3, positive control, which contained CYP2C19 RNA only without competitor RNA; lanes 4–8, 0.28 fg CYP2C19 RNA plus fourfold dilutions (2.66, 0.67, 0.17, 0.042 and 0.01 fg) of competitor RNA.

Table 1. Reproducibility of Full-Length CYP2C19 RNA Assay

	CYP2C19 RNA detected in liver (copies $\times 10^5/\mu\text{g}$ liver RNA)			
	L1	L2	L3	L4
<i>Intraday variation</i>				
Replicate 1	27.0	5.1	4.1	3.5
Replicate 2	23.6	3.8	3.9	2.6
Replicate 3	24.7	4.6	3.8	3.3
Replicate 4	18.0	3.5	4.2	3.9
Mean	23.3	4.3	4.0	3.3
SD	3.8	0.8	0.2	0.5
CV(%)	16.4	18.1	4.4	16.5
<i>Interday variation</i>				
Day 1	18.0	3.5	4.2	3.9
Day 2	24.3	5.1	3.3	3.2
Day 3	29.7	5.6	4.5	2.8
Mean	24.0	4.7	4.0	3.3
SD	5.9	1.1	0.6	0.5
CV(%)	24.4	23.5	16.1	15.9

Quantitation and Characterization of Full-Length CYP2C19 RNA in Human Livers

The total RNA from livers of known genotypes and *S*-mephenytoin-4'-hydroxylase activities were analyzed for the full-length CYP2C19 RNA. A typical gel analysis of this assay is shown in Figure 3, and the data for liver samples are summarized in Table 2. The CYP2C19 RNA transcript ranged from $0.1\text{--}23 \times 10^5$ copies/ μg liver RNA in the 10 human samples tested. A preliminary linear regression analysis of RNA vs. *S*-mephenytoin-4'-hydroxylase activity in microsomes yielded an *r* value of 0.54. When liver 8 was considered as an outlier, the *r* value increased to 0.78. Because the small numbers of our liver samples may not be representative of the population, and the lack of *3/*3 genotypes, these correlation data should be considered tentative.

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Table 2. Characterization of 11 Livers in Terms of Genotype, CYP2C19 RNA (Copies/μg Liver RNA) and Enzyme Activity

Liver ID	Genotype ^a	CYP2C19 RNA Detected in Liver (copies × 10 ⁵ /μg liver RNA)	Enzyme Activity ^b (pmol/min/mg)
1	*1/*1	23.3	13.4
2	*1/*1	4.3	7.8
3	*1/*1	4.0	4.8
4	*1/*1	3.3	3.0
5	*1/*1	0.1	0.8
6	*1/*2	7.8	2.7
7	*1/*2	0.3	2.3
8	*1/*2	0.4	12.7
9	*1/*2	0.1	5.7
10	*1/*2	0.1	6.4
11	*2/*2	0.0	0.0

^aGenotype, genomic DNA from corresponding livers were subjected to PCR using *2-specific primers, and followed by *Sma*I digestion. For *2, the point mutation G→A is located in exon 5 leading to a premature stop codon and a truncated 234 amino acid protein. This mutation is found in poor metabolizers of *S*-mephenytoin. *1/*1, wild-type.

^bCYP2C19 enzyme activity was determined by incubating 50 μM *S*-mephenytoin with human liver microsomes and quantitating the formation of the 4'-hydroxylated product.

To determine whether the RNA transcript detected from the heterozygous (wt/*2) individuals contained both variants of the allele, we subjected the DNA products from RT-PCR to restriction fragment analysis. The expected *Sma*I-digested products for the *1 allele are 800-bp and 680-bp fragments, while the DNA product of the *2 allele should be resistant to enzyme digestion. Thus, the RT-PCR products of the *1/*2 genotype should consist of three

bands: 1440, 800 and 680 bp. However, we found that the products from the *1/*2 genotype were completely digested by *Sma*I into two 800-bp and 680-bp fragments, identical to that of the wt (*1/*1) RT-PCR products (Figure 4, panel A). These results suggest that the aberrant *2 allele of the *1/*2 genotype either did not produce full-length CYP2C19 transcript or produced it at an undetectable level.

To further verify that the unde-

tectable full-length CYP2C19 transcript of the *2 allele was not caused by RNA preparation or the RT-PCR procedure used for the assay, we used the same RNA preparation and RT-PCR reagents to produce much shorter DNA products from a small but critical region of RNA, according to the method of de Morais et al. (11). With this RT-PCR method, a 284-bp product is expected for the wt/wt genotype, while a 40-bp deleted, 244-bp product is expected for the aberrant *2/*2 genotype (11). As shown in Figure 4, panel B, both 284-bp and 244-bp bands were detected for the RNA samples from the *1/*2 genotypes, while only a single 284-bp product was observed for livers of the *1/*1 genotype.

Collectively, these data suggest that while a small region (<500 bp) of *2 transcripts can be detected with nearly equal frequency to that of the *1 copy in liver RNA of *1/*2 heterozygous genotypes, the full-length transcripts of these genetic variants may be expressed at a much lower level than that of the *1 transcripts.

DISCUSSION

RT-PCR has been used successfully to quantitate human immunodeficiency

Figure 2. Northern analysis of CYP2C19 RNA. Varying amounts of CYP2C19 RNA were separated and then analyzed by Northern analysis using the cDNA probe as described in Materials and Methods. Calculated RNA copy number is based on the amount of RNA loaded in each lane (1–4). The estimated RNA copy number is experimentally derived from the mean value of duplicate RT-PCR assay determinations.

Figure 3. Quantitation of CYP2C19 RNA in three representative livers using quantitative competitive RT-PCR assay. Serial dilutions of total RNA from livers were reverse transcribed and amplified with a constant amount (2700 copies) of competitor RNA. The RT-PCR products were separated on a 1% agarose gel and stained with ethidium bromide. Lane 1: 1-kb DNA ladder; lane 2: CYP2C19 standard RNA (2700 copies) and competitor RNA; lanes 3–5 and lanes 6–8: the products of total RNA from liver 6 (11, 3.5 and 1.4 ng total RNA) and liver 3 (15, 5 and 1.6 ng total RNA); lane 9: the products of 500 ng RNA from liver 11 (*2/*2).

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virus (25,37), hepatitis C virus (20,21) and the transcripts of CYP P450 RNA molecules (1,2,23,24,26). Previously, endogenous RNA standards such as β -actin and GAPDH sequences were used as internal controls to estimate relative levels of CYP2E1 mRNA (26) and CYP1B1 (5) using RT-PCR. Endogenous internal standards, presumed to be invariant, often exhibit widely differing abundance, differing amplification efficiency and varying sensitivity to some experimental treatments (14). The homologous, external synthetic RNA standard (34) shares the same primer binding sites as the native RNA and has the same intervening sequence except for a small insertion or deletion as the native RNA. The homologous RNA standard used in both RT and PCR steps with an identical set of primers is most likely to have the same or very similar RT and PCR efficiencies. This strategy has been used to detect RNA sequences of

CYP1A1 (33), CYP1B1 (10), CYP1A2, CYP2A6/7, CYP2D6, CYP2E1 and CYP3A4/5/7 (2).

However, the RT-PCR assays used in these studies produced short fragments that were less than 680 bp. None of the above studies estimated the amount of the full-length gene products, the basis for expression of proteins. While it identifies part, but not all, of the open reading frame, a small RNA fragment detection method may overestimate the full-length RNA transcripts that are required to express protein. This is because total RNA may be partly degraded, enabling detection by the short RT-PCR method. With the improvements in RNA isolation (to consistently obtain high-quality RNA) and in reverse transcriptase and polymerase enzymes used in PCR technology, it is now possible to measure the full-length CYP P450 RNA molecules in a reproducible manner. Indeed, we have de-

monstrated the feasibility of using a modified CYP2C19 RNA as a competitor RNA to detect and estimate the full-length CYP2C19 RNA in human livers using quantitative competitive RT-PCR technology. This assay permits a semi-quantitative estimation of CYP2C19 full-length RNA found in different samples of liver tissue.

Whether this RT-PCR method described here can be used to estimate CYP2C19 RNA transcripts in blood is still unclear. Because it is very sensitive and specific, one should be able to detect full-length CYP2C19 RNA sequences if the gene is transcribed in blood cells such as lymphocytes. Analysis of CYP2D6 mRNA with RT-PCR in blood lymphocytes of extensive metabolizers of debrisoquine ($n = 12$) produced a significant correlation between CYP2D6 mRNA levels in circulating peripheral blood mononuclear cells and the *in vivo* debrisoquine

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Figure 4. Characterization of CYP2C19 RNA transcripts using DNA products isolated from the long and short RT-PCR methods. The RNA isolated from identical livers were subjected to RT-PCR using different primers to obtain long (Panel A) and short (Panel B) CYP2C19 DNA. Panel A: The genotype variation of mRNA expressed was determined by subjecting the DNA product to restriction enzyme treatment ($\pm$ *Sma*I). The products of RT-PCR and their respective digestion products were analyzed on a 1% agarose gel and stained with ethidium bromide. Data from four representative livers are shown. Lane 1: 1-kb DNA ladder. Panel B: Short RT-PCR analysis to detect the *2: exon 5 aberrant gene and to compare RNA expression of genotype variations. The products were separated on a 3% agarose gel. Lane 1: 100-bp DNA ladder.

recovery ratio ($r = 0.724$) (8). If quantitation of the CYP2C19 RNA levels in lymphocytes predicts the enzyme activity, one could potentially use this information to determine the proper dose to add that would allow maximum therapeutic efficacy and minimum toxicity.

During the course of RNA analysis, we unexpectedly found that no full-length CYP2C19 RNA was detectable in the liver of the *2/*2 genotype (Figure 3, lane 9). Further analysis of CYP2C19 transcripts from liver of *1/*2 genotypes based on restriction enzyme analysis of the RT-PCR product suggests that only normal (*1), and not the variant *2 copy of full-length CYP2C19 RNA, was present in these livers. When the same RNA samples were subjected to a less demanding short RT-PCR, both *1 and *2 RNA sequences were detected (Figure 4, panel B). It is possible that while both the normal and aberrant copies of a gene can be transcribed, the full-length RNA of the *2 variant allele may be transcribed at a much lower efficiency such that its presence can be detected only in a less demanding short RT-PCR assay. Alternatively, both RNA transcripts are transcribed with equal efficiency; however, the aberrant transcript is intracellularly degraded at much higher rates, thereby producing similar results. In contrast, for CYP2C9, about five- to tenfold higher RNA transcript levels of mutant alleles than the normal alleles were detected in heterozygous individuals (7).

While the detailed regulation of CYP2C19 and translation into a functional product remains elusive, the assay described here should allow one to systematically address the synthesis and fate of full-length CYP2C19 in a reproducible manner. The assay needs only a very small amount of tissue (approximately 10 mg) to isolate 20–50 μ g RNA. Although we routinely use 500 ng total RNA per assay to detect CYP2C19 RNA in livers for initial screening and subsequent titration to estimate CYP2C19 RNA copies, this assay needs only 10–100 ng total RNA when the CYP2C19 RNA concentration is about 10^5 copies/ μ g RNA. While the assay described is for liver RNA samples, the method can readily be adopted to detect RNA isolated from other tissues and blood cells.

In summary, we have developed an internally controlled semi-quantitative competitive RT-PCR assay to detect full-length CYP2C19 RNA transcripts. This method can be used to develop assays for the detection of full-length RNA transcripts of other CYP enzymes as well as other drug-metabolizing enzymes and transporters.

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