Quantification of the Levels of *CYP2D6* mRNA in Peripheral Blood Leukocytes by Reverse Transcriptase Polymerase Chain Reaction HPLC

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Received January 20, 2009; accepted September 3, 2009; published online September 17, 2009

We developed a sensitive high performance liquid chromatography (HPLC) method for determining *CYP2D6* mRNA in peripheral blood leukocytes (PBL) by using competitive reverse transcriptase polymerase chain reaction (RT-PCR). The method is specific, reproducible, and sensitive enough to quantify the absolute amount of low and high abundant *CYP2D6* mRNA. The native *CYP2D6* transcript and the internal standard, a *CYP2D6* deletion RNA, were amplified with similar efficiency in RT-PCR. The PCR products were separated as the corresponding peaks in optimized HPLC. The coefficients of variation for competitive RT-PCR and HPLC determination were 1.5—6.5% and 0.6—2.4%, respectively, showing high reproducibility and reliability. This approach could also be applicable to the quantification of mRNA expressing on various tissues, including PBL, of which the expression levels were so low that they were hard to determine by existing agarose gel electrophoresis methods.

Key words *CYP2D6*; peripheral blood leukocyte mRNA; high performance liquid chromatography

Several methods have been reported for the semi-quantitative detection of small amounts of a particular mRNA in tissues through specific amplification of the extracted mRNA by reverse transcriptase polymerase chain reaction (RT-PCR), usually followed by agarose gel electrophoretic separation and ethidium bromide staining for fluorescence detection.[1—4] However, most of the methods are not suitable for accurate quantification owing to lack of correction for incomplete recovery of mRNA during extraction from the tissue or subsequent PCR procedures. It is also difficult to avoid PCR amplification into a non-linear region because of insufficient detection sensitivity. To overcome these problems, competitive RT-PCR with an internal standard has been shown to be a useful method for mRNA quantification, where high performance liquid chromatography (HPLC) with a UV detector was employed for detecting PCR products.[5—7]

CYP2D6 with genetic polymorphism is involved in the oxidative metabolism of many drugs including neuroleptics, tricyclic antidepressants, selective serotonin reuptake inhibitors, β-adrenoreceptor blockers, and antiarrhythmics.[8,9] Although the genotyping of *CYP2D6* is useful for assessing drug efficacy and adverse effects, it is hard to conduct routine clinical measurement because of the many single nucleotide polymorphisms (SNPs). Since blood is a readily accessible tissue, an appealing concept should be to use peripheral blood leukocytes (PBL) as a surrogate for CYP activities that manifest in internal organs.[10—12] In the present study, we developed a sensitive and quantitative RT-PCR-HPLC method for the measurement of *CYP2D6* mRNA in PBL.

Experimental

Preparation of RNA Standards for RT-PCR *wt* 2D6, the standard cDNA containing 456 bp of *CYP2D6* DNA (nt 1087—1542), was cloned to pBluescript KS (+) vector by PCR from cDNA Library Human Liver (Takara Bio, Japan), and designated as pKSp2D6. For preparation of the internal standard, *is 2D6*, 68 bp (nt 1107—1153) was deleted from pKSp2D6 using PCR, and named as pKSd2D. The primers used for amplification of *wt 2D6* were CGAGCAAGCTT-Ex7F, 5′-CGAGCAAGCTTGGAGATCGACGACGTGATAG-3′ and AGGTCGTCGAC-Ex9R, 5′-AGGTCGTCGACACCAGGAAAGCAAAGACACC-3′. The primers used for amplification of *is 2D6* were CGAGCAAGCTT-Ex7F D46, 5′-CGAGCAAGCTTGGAGATCGACGACGTGATAGGTGCAGCGCTTTGGGGACGACAT-3′ and AGGTCGTCGAC-Ex9R, 5′-AGGTCGTCGACACCAGGAAAGCAAAGACACC-3′. The standard RNAs, *wt 2D6* and *is* 2D6, were prepared using Riboprobe® *in vitro* Transcription Systems (Promega, U.S.A.) from pKSp2D6 and pKSd2D6, respectively. The RNAs were treated with RNase-free DNase (QIAGEN, Netherlands) and purified by QIAamp® RNA Blood Mini (QIAGEN). The RNA standards were diluted to the concentration of 1 pg/μl and stored in aliquots at −80 °C until use.

Subjects The study was explained to 8 healthy volunteers (2 male and 6 female) and written informed consent was obtained from each. The study was approved by the Institutional Review Board of the Faculty of Pharmaceutical Sciences, Teikyo University and University of Tsukuba.

Extraction of RNA from Human Blood Cells Whole blood (1.5 ml) was collected from the subjects in an EDTA-2Na-containing Leukoprep tube (Terumo, Japan) at room temperature. Total RNA was isolated using a QIAamp RNA Blood Mini (QIAGEN). The purity and quality of RNA were determined spectrophotometrically (Amersham Pharmacia Biotech, U.S.A.). The RNA samples were treated with RNase-free DNase I (QIAGEN) to digest any contaminating genomic DNA. The OD 260/280 ratios for quantitative RNA quality assessment were 1.929±0.0465 (n=9) and they met the general requirements.

cDNA Synthesis cDNA synthesis was performed using an RNA PCR Kit (AMV) Ver.3.0 (Takara Bio). Two hundred picograms of total cellular RNA or 1 pg of *wt* 2D6 RNA was added to 10 μl of the RT reaction mixture with the internal standard, 1 pg of *is* 2D6 RNA, which consists of 20 mM Tris–HCl (pH 8.3), 2.5 mM $MgCl_2$, 1 mM deoxynucleotide triphosphates (dNTPs), 10 units of RNase inhibitor, 0.125 μM oligo(dT) primer, and 2.5 units of avian myeloblastosis virus (AMV)-XL reverse transcriptase. The mixture was incubated at 42 °C for 15 min, heated at 99 °C for 5 min, and put on ice. The cDNAs were then stored at −20 °C.

PCR Conditions PCR was performed by adding 10 μl of cDNAs to a total volume of 50 μl of 1×PCR buffer, 1 mM Ex7f and Ex9R primers, and 1.25 units of TaKaRa Taq HS™ (Takara Bio). Amplification of *CYP2D6* DNA was carried out for 27 cycles using Thermal Cycler SP (Takara Bio). The initial one cycle consisted of a denaturation step at 95 °C for 2 min. The remaining 26 cycles each consisted of a denaturation step at 95 °C for 2 min, an annealing step at 65 °C for 30 s, and an elongation step at 72 °C for 3 min. The primers used for amplification of *CYP2D6* DNA from *wt 2D6*, *is 2D6*, and the samples of the subjects were Ex7F, 5′-GGAGATCGACGACGTGATAG-3′ and Ex9R, 5′-ACCAGGAAAGCAAAGACACC-3′.

HPLC Aliquots of 10 μl of the PCR products were injected manually into a TSK-gel DNA-NPR, 4.6 mm i.d.×75 mm column (Tosoh, Japan) every 28 min. The PCR products were separated by linear gradient elution

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with NaCl (from 0.55 to 0.6 M over 10 min) in 20 mM Tris–HCl (pH 9.0) at a total flow rate of 0.75 ml per min delivered from LC-10 AD pumps (Shimadzu, Japan). The eluent was in-line-filtered with a TSK-guardcolumn PWXL, 6.0 mm i.d.×40 mm column (Tosoh). The eluate was monitored at 260 nm with a UV detector connected to an integrator. The absorbance unit full scale (AUFS) of the detector was normally set to 0.004. The peak areas of PCR products derived from cellular RNA, standard *wt 2D6* RNA, and internal standard *is 2D6* RNA were calculated by an integrator. The amount of mRNA in samples was estimated by the comparison of sample-to-internal standard ratios with a standard RNA-to-internal standard ratio.

Results and Discussion

Optimal Conditions for Quantification of *CYP2D6* mRNA by RT-PCR-HPLC *wt 2D6*, encoding CYP2D6 cDNA (nt 1087—1542) and *is 2D6* DNA fragments with deletion of 68 bp (nt 1107—1153) from *wt 2D6* were purified by using agarose gel (MinElute Gel Extraction kit) and 10 ng of the DNA was then injected into the HPLC. A typical chromatogram for detecting *wt 2D6* and *is 2D6* is presented in Fig. 1. The retention times of *is 2D6* (388 bp) and *wt 2D6* (456 bp) were 17.3 and 17.9 min, respectively. The resolution of the column is high, as indicated by separation of the two closely-related size bands. This technique lends itself ideally to the use of PCR for quantitative gene expression measurements. To determine the cycle number of PCR suitable for exact quantification of *CYP2D6* mRNA, 100 fg of *wt 2D6* RNA and internal standard, *is 2D6* RNA, were reverse-transcribed with oligo(dT) primer and amplified by PCR with *CYP2D6* specific primers. The cycle number dependency of PCR products was checked in the range of 24—30 cycles. The efficiency of PCR decreased gradually when the cycle number exceeded 28. Therefore, we set the number of PCR cycles to 26 (data not shown). To examine whether amplification efficiency is dependent on the initial amount of RNAs, equal amounts of *wt 2D6* and *is 2D6* from 1 to 100 fg were added to one tube and amplified in the RT reaction mixture. The amount of RT-PCR products was proportional to the added amounts of *wt 2D6* and *is 2D6* in the range up to 10 fg/tube (Fig. 2). Linearity was lost over 10 fg with both standard RNAs. The calibration curve was linear in a practical range of measurements in the RT-PCR-HPLC. Values could be corrected for RNA recovery during extraction and the absolute PBL levels of mRNA could be determined, since an internal standard was used in the method. Furthermore, equal efficiencies of amplification were obtained because the same primers were used for target mRNA and internal standard RNA. The detection limit of *CYP2D6* mRNA in PBL under 26 cycles of amplification was 0.1 fg/100 ng RNA and could be enhanced by increasing the numbers of PCR cycles.

Accuracy and Reproducibility of *CYP2D6* mRNA Quantification by RT-PCR-HPLC To compare the reproducibility in measuring RT-PCR products between two methods, HPLC and agarose gel electrophoresis, mixtures of *is 2D6* and *wt 2D6* RNA were subjected to RT-PCR in the ratios of 2 : 1, 1 : 1, or 1 : 10, or 2 : 1, 1 : 1, or 1 : 5, respectively. The PCR products were quantified using agarose gel electrophoresis (Table 1) or HPLC (Table 2). RT-PCR was performed 3 times (a, b, c) and the quantification of each PCR sample was carried out in triplicate. The within-sample coefficient of variation was 3.6—35.9% for agarose gel electrophoresis (Table 1) and 0.6—2.4% for HPLC (Table 2). The PCR standard deviation was 13.8—23.6% for agarose gel electrophoresis (Table 1) and 1.5—6.5% for HPLC (Table 2). These results demonstrate that HPLC is more reliable than agarose gel electrophoresis for quantification of *wt 2D6* RNA. The limit of sensitivity was reached at 1 ng of *CYP2D6* PCR product injected into the HPLC. On the other hand, a minimum of 50 ng of DNA could be detected by ethidium bromide staining and direct examination of the gel under UV. Therefore, this new application provides a 50-fold sensitivity advantage over agarose gel electrophoresis. Another advantage is the speed of the analysis, since *CYP2D6* and internal standard peaks were resolved within 18 min, allowing 60 samples to be run during the night with an automatic injector. Thus, a large number of clinical samples can be evaluated by the RT-PCR HPLC method.

***CYP2D6* mRNA Levels in Blood Cells by RT-PCR-HPLC** Two hundred nanograms of total RNA isolated from PBL was added to the RT-PCR reaction mixture with 1 fg of *is 2D6* RNA, an internal standard. A typical chromatogram for PBL samples is shown in Fig. 3. The peak of *CYP2D6* mRNA derived from PBL was detected at a similar retention time when *wt 2D6* DNA fragment purified by gel extraction was used (Fig. 1). To quantify *CYP2D6* mRNA in PBL, 200 ng of cellular RNA was used with 1 to 10 fg of *is 2D6* (Fig.

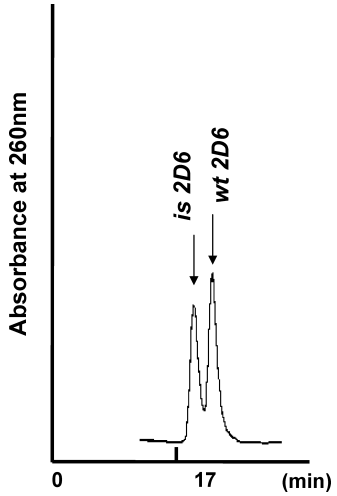

Fig. 1. Representative HPLC Tracing of *wt 2D6* and *is 2D6* DNA

wt 2D6 (456 bp) and *is 2D6* DNA (388 bp) fragments were purified from agarose gel and 10 ng of each DNA was injected into the HPLC apparatus.

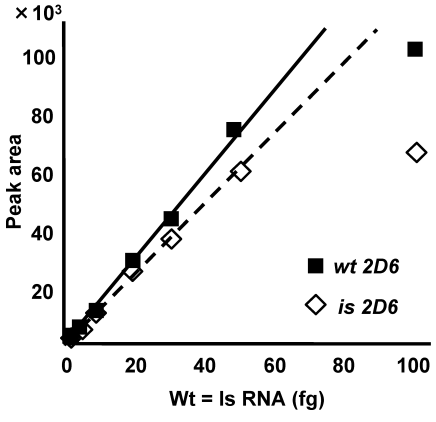

Fig. 2. The Dependency of RT-PCR Products on Initial Amounts of Standard RNAs

Various amounts of *wt 2D6* and internal standard, *is 2D6* RNAs, were reverse-transcribed with oligo(dT) primer into cDNA. They were amplified by PCR for 26 cycles with *CYP2D6* specific primers and analyzed by HPLC with a UV detector (260 nm).

Table 1. Accuracy and Reproducibility of Quantification RT-PCR Products by Agarose Gel Electrophoresis

PCR sample	Approximate ratio, Is : Wild[a)]								
	2 : 1			1 : 1			1 : 10		
	Ratio (Is/Wild)	AE[b)] stdev (%)	PCR stdev (%)	Ratio (Is/Wild)	AE stdev (%)	PCR stdev (%)	Ratio (Is/Wild)	AE stdev (%)	PCR stdev (%)
a	2.044	0.373	0.494	0.950	0.189	0.170	0.064	0.007	0.001
	1.654	(18.4%)	(23.6%)	1.118	(16.7%)	(13.8%)	0.051	(12.4%)	(20.3%)
	2.400			1.327			0.062		
b	1.831	0.757		1.226	0.199		0.059	0.016	
	1.533	(35.9%)		1.040	(16.2%)		0.028	(35.7%)	
	2.968			1.439			0.052		
c	1.683	0.502		1.209	0.118		0.050	0.002	
	2.072	(23.4%)		1.300	(8.9%)		0.052	(3.6%)	
	2.680			1.442			0.054		

a) *is* 2D6(388) : *wt* 2D6(456)=10 fg : 5 fg, 5 fg : 5 fg, 5 fg : 50 fg=2 : 1, 1 : 1, 1 : 10. *b*) AE; agarose gel electrophoresis.

Table 2. Accuracy and Reproducibility of Quantification RT-PCR Products by HPLC

PCR sample	Approximate ratio, Is : Wild[a)]								
	2 : 1			1 : 1			1 : 5		
	Ratio (Is/Wild)	HPLC stdev (%)	PCR stdev (%)	Ratio (Is/Wild)	HPLC stdev (%)	PCR stdev (%)	Ratio (Is/Wild)	HPLC stdev (%)	PCR stdev (%)
a	2.207	0.034	0.033	1.299	0.023	0.083	0.198	0.002	0.007
	2.144	(1.6%)	(1.5%)	1.299	(1.8%)	(6.5%)	0.197	(1.0%)	(3.5%)
	2.198			1.260			0.201		
b	2.203	0.017		1.394	0.033		0.210	0.004	
	2.228	(0.8%)		1.364	(2.4%)		0.204	(1.7%)	
	2.194			1.328			0.204		
c	2.234	0.013		1.205	0.008		0.211	0.002	
	2.250	(0.6%)		1.188	(0.7%)		0.212	(1.0%)	
	2.259			1.194			0.213		

a) *is* 2D6(388) : *wt* 2D6(456)=10 fg : 5 fg, 5 fg : 5 fg, 5 fg : 25 fg=2 : 1, 1 : 1, 1 : 5.

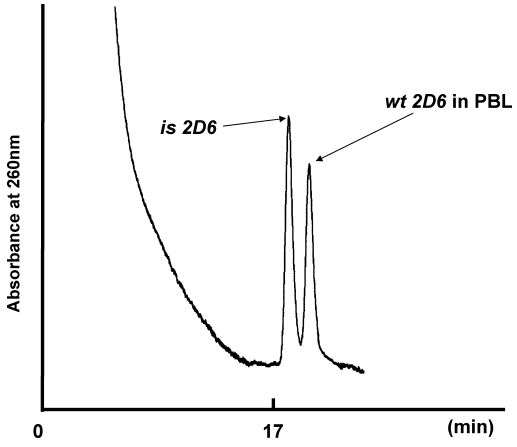

Fig. 3. Representative HPLC Tracing of *CYP2D6* mRNA in PBL

Two hundred nanograms of total RNA extracted from PBL was reverse-transcribed with 1 fg of *is 2D6* DNA and amplified by PCR for 26 cycles with *CYP2D6* specific primers, followed by HPLC analysis with a UV detector (260 nm).

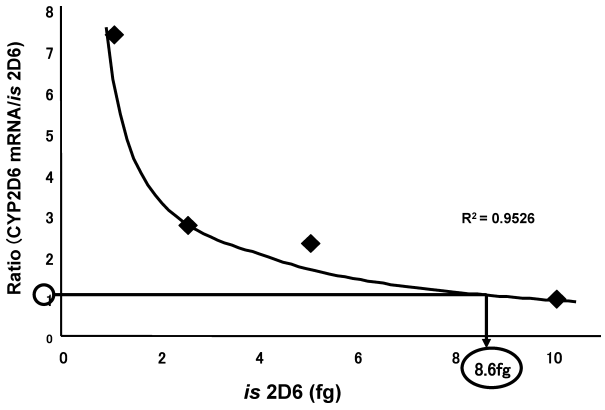

Fig. 4. Quantification of *CYP2D6* mRNA in PBL

Two hundred nanograms of cellular RNA extracted from PBL was probed using similar methods with decreasing amounts of *is 2D6*, internal standard RNA from 1 to 10 fg.

4). We detected 8.60 fg of *CYP2D6* mRNA in 200 ng of cellular RNA in PBL, since the amplification efficiencies of *wt 2D6* and *is 2D6* are similar in our RT-PCR-HPLC (Fig. 2). To evaluate the linearity between the added amounts of cellular RNA and levels of *CYP2D6* mRNA, increasing amounts of total RNA up to 400 ng were added to the reaction mixture with 5 fg of *is* 2D6. As shown in Fig. 5, a linear relationship (r^2=0.9944) was observed between the levels of *CYP2D6* mRNA and the cellular RNA in a range of 50—400 ng. Our RT-PCR-HPLC method was used to determine *CYP2D6* mRNA in RNA samples isolated from 8 healthy PBL. The levels of *CYP2D6* mRNA/total RNA 100 ng were between 0.34 and 3.92 fg (Table 3). This suggests that our RT-PCR-HPLC method would be efficient for the accurate quantifica-

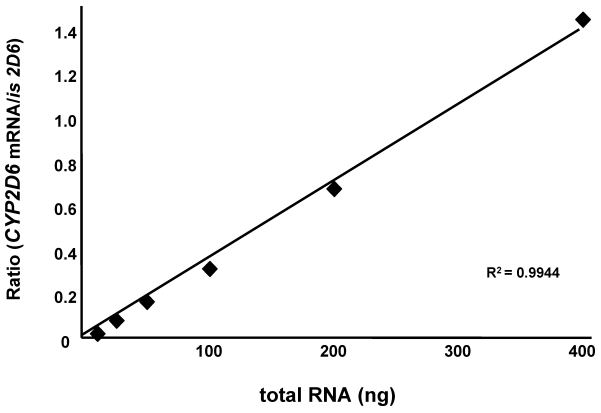

Fig. 5. Calibration Curve

Various amounts of total cellular RNA in the range of 20—400 fg together with 5 fg of *is 2D6* were reverse-transcribed and amplified by PCR for 26 cycles.

Table 3. *CYP2D6* mRNA Levels in PBL of Healthy Volunteers by RT-PCR-HPLC

Healthy volunteer No.	Total RNA (ng)	*is*2D6 (fg)	*wt*/*is*	fg/100 ng
101	99	1	0.341	0.34
102	171	1	0.492	0.29
103	139.5	1	0.385	0.28
107	272.7	5	2.141	3.92
115	117	1	1.655	1.41
116	137.7	1	1.507	1.09
117	152.1	1	0.535	0.35
118	121.5	1	0.586	0.48

tion of *CYP2D6* mRNA levels in PBL. Recently, the quantification of mRNA levels of several genes in PBL by real-time PCR has been reported.[23—25] Although real-time PCR is a powerful technique for estimating low levels of mRNA from many samples at a time, it is difficult to correct for RNA recovery during extraction using the method. In real-time PCR, as a reference for RNA levels, endogenous housekeeping genes such as β-actin and GAPDH are often used. However, the expression levels of these genes vary in different conditions and separate analyses with different numbers of cycles of PCR are required if the levels are quite distinct from the target gene levels.[26] On the other hand, the amount of an exogenously added internal standard, which is used in our method, can be adjusted according to the level of mRNA in samples, thereby allowing measurement in the same tube.

In conclusion, the RT-PCR-HPLC method presently described for estimating the amounts of *CYP2D6* mRNA in PBL is a valuable analytical approach as it is specific, sensitive, and clinically applicable. Although it remains unclear whether *CYP2D6* mRNA levels in PBL are correlated with hepatic CYP2D6 enzyme activity, the present method could be useful for elucidating this clinically important subject. This approach could also be applied to the determination of low mRNA levels of other significant *CYPs*, which may vary according to genetic and environmental parameters.

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