were (1.07–1.11) and (10–65), respectively. The SD of residuals \( S_{ij} \) was 73 ng/L in the concentration range of 0–5000 ng/L and 22 ng/L in the range of 0–500 ng/L. The agreement between the GC/MS and MEIA assays indicated low interference from other estrogenic compounds and their metabolites in the latter assay.

In conclusion, we have developed a GC/MS method for the quantification of estradiol in patient serum samples and serum-based immunoassay calibrators; the method was validated by use of CRMs. The utility of the method was further demonstrated by the presented correlation between estradiol concentrations in patient samples measured by MEIA and GC/MS.

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References

Rapid Quantification of CYP3A4 Expression in Human Leukocytes by Real-Time Reverse Transcription-PCR, Isabella Nowakowski-Gashaw,1 Przemyslaw M. Mrózkiwietz,1 Ivar Roots1, and Jürgen Brockmüller1,2* (1 Institute of Clinical Pharmacology, Charité University Medical Center, Humboldt University of Berlin, 10098 Berlin, Germany; 2 Department of Clinical Pharmacology, University Medical Center, Georg-August University of Göttingen, 37075 Göttingen, Germany; * address correspondence to this author at: Department of Clinical Pharmacology, University Medical Center, Georg-August-University of Göttingen, Robert-Koch-Strasse 40, 37075 Göttingen, Germany; fax 49-551-3912767, e-mail jürgen.brockmoller@med.uni-goettingen.de)

Cytochrome P450 3A4 (CYP3A4) contributes to the metabolism of a wide variety of drugs and endogenous substrates, such as steroid hormones (1, 2). Variations in the catalytic activity of CYP3A4 are predominantly caused by enzyme induction mediated by transcriptional activation or by competitive substrate inhibition. Such variation may strongly influence the bioavailability of drugs and may modulate drug interactions. CYP3A4 is one of the predominant CYPs in the human liver, accounting for ~30% of the total hepatic cytochrome P450 protein (2, 3). Relatively high CYP3A4 concentrations have been found in the small intestinal epithelium (70% of total CYP protein) and in the kidney (2). There are conflicting results concerning the amount of CYP3A4 in human peripheral blood lymphocytes. Several authors could not detect any CYP3A4 mRNA or protein, whereas some studies reported poor CYP3A4 expression in the white cell fraction (4–6). Thus, we assumed that CYP3A4 is expressed in lymphocytes in very small amounts and that only a very sensitive method could detect them. We developed a sensitive quantitative real-time reverse transcription-PCR (RT-PCR) method that allows rapid and correct determination of CYP3A4 mRNA expression in leukocytes.

We investigated CYP3A4 mRNA expression in 31 human blood samples from healthy volunteers (20 males and 11 females; mean age, 29 years; range, 20–64 years) and in three human liver samples obtained from the International Institute for the Advancement of Medicine (Exton, PA). Before blood collection, all volunteers signed informed consents that were accepted by the Ethical Committee of the Charité. Leukocytes were separated from 8 mL of whole blood in a Vacutainer® opt cell preparation tube system (Becton Dickinson). Small liver fragments were disrupted with a homogenizer (Potter; Braun). Samples were stored at −80 °C. Total cellular RNA was extracted by the TRizol® LS method according to the manufacturer’s protocol (Life Technologies). The procedure was modified by the addition of 10 ng of RNase-free glycogen (Roche) before homogenization. The RNA solution was digested with 10 U of RNase-free RQ1 DNase (Promega) for 15 min at 37 °C to exclude genomic DNA from the preparation; extraction with TRizol LS was then repeated. RNA concentration and purity were deter-
The mRNA was first transcribed into cDNA; a fragment of CYP3A4 was then amplified. Reverse transcription was performed using Moloney murine leukemia virus reverse transcriptase in a PE9700 thermocycler (Applied Biosystems). Total cellular RNA (100 ng) was added to the reverse transcriptase in a PE9700 thermocycler (Applied Biosystems). Reverse transcription was performed using Moloney murine leukemia virus reverse transcriptase in a PE9700 thermocycler (Applied Biosystems). The fragment, with a length of 170 bp, was designed to span intron 12 (genomic DNA sequence EMBL AF209389). The forward primer was situated in exon 12, whereas the reverse primer was in exon 13 of the CYP3A4 gene to prevent detection of any possible contamination by genomic DNA. To check the specificity of the chosen oligonucleotides, we sequenced the obtained amplicons, and only CYP3A4 sequences were amplified. Reverse transcription was achieved.

To calibrate the expression of the CYP3A4 gene, we constructed an externally added standard. The external standard was designed to be identical in size and sequence to the CYP3A4 fragment. We therefore carried out a conventional RT-PCR with the primers CYP3A4F and CYP3A4R, as described above, and purified the cDNA amplicons, diluted in 20 μL of water, with Ultrafree MC 30 000 filters (Millipore) according to the manufacturer’s protocol. In the subsequent PCR, the CYP3A4 amplicon was prepared for in vitro transcription. The purified amplicon (4 μL) was added to the reaction mixture containing 20 μL/L Tris (pH 8.4), 50 mmol/L KCl, 2 mmol/L dithiothreitol, 1 mmol/L dNTPs, 2 μmol/L oligo(dT)15 primer, 0.5 μg of bovine serum albumin, 8 units of RNAsin (Promega), and 120 U of Moloney murine leukemia virus reverse transcriptase (Life Technologies). The reaction mixture was incubated for 60 min at 42 °C, and then heated for 2 min at 95 °C, followed by rapid cooling to 4 °C. The obtained transcripts were stored at −20 °C.

cDNA was amplified in a final volume of 20 μL containing 20 mmol/L/L Tris (pH 8.4), 50 mmol/L KCl, 0.5 mmol/L MgCl2, 50 μmol/L dNTPs, 1 μmol/L each of the primers CYP3A4F (5′-CTTATATATACACACCCCTTGGGAAAG-3′) and CYP3A4R (5′-GGTGAGAAGATCCCTCCTAAGCT-3′), and 1 U of Platinum® Polymerase (Life Technologies). Lastly, 8 μL of cDNA (corresponding to 40 ng of total RNA) was added from the reverse transcription. Cycling conditions were 2 min at 94 °C and 30 s at 94 °C, 55 °C, and 72 °C for 40 cycles each, followed by 4 min at 72 °C. A 5-μL aliquot of the amplicons was separated on an agarose gel.

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After the PCR product was separated on an agarose gel, it was purified with Ultrafree MC 30 000 filters. The RNA was then synthesized in a 90-min in vitro transcription step at 37 °C with the DNA-dependent T7 RNA polymerase (Promega) according to the manufacturer’s protocol. After digestion with RNase-free RQ1-DNase, the quality of the transcripts was checked by gel electrophoresis, and
the transcripts were extracted with phenol–chloroform. The amount of CYP3A4 standard RNA was calibrated by absorbance at 260 nm, and the specificity was confirmed by sequencing.

CYP3A4 was quantified on a LightCycler™ (Roche). Transcribed cDNA (40 ng) was added to a total volume of 15 μL containing 14 mmol/L Tris (pH 8.4), 35 mmol/L KCl, 50 μmol/L dNTP mixture, 3 mmol/L MgCl₂, 0.6 μg of bovine serum albumin, 1 mL/L dimethyl sulfoxide, 6 pmol of each primer (CYP3A4F and CYP3A4R), 3 pmol of each hybridization probe (CYP3A FLU, 5'-AGTTTCAT-GTTCACGAGAGCAAACCTC-fluorescein; and CYP3A LCR, 5'-LCRed640-TGCCAAATGCAGTTTCTGGGTCGCA), and 1.65 U of Platinum Taq Polymerase (Life Technologies). Samples were then amplified by 45 cycles of repeated denaturation (0 s at 95 °C, with a ramp rate of 20 °C/s), annealing (10 s at 55 °C, with a ramp rate of 20 °C/s), and enzymatic chain extension (10 s at 72 °C, with a ramp rate of 2 °C/s). The fluorescence was measured at the end of each annealing step. Serial dilutions of CYP3A4 external standard RNA were added to each charge of reactions and amplified during each run in separate capillaries. A calibration curve was automatically generated from the external standards.

The raw data were evaluated with the LightCycler run software, Ver. 5.32. The absolute copy number of CYP3A4 transcripts in samples was calculated using a fluorescence calibration curve obtained from amplicons of serially diluted standard RNA that was synthesized by in vitro transcription. The threshold cycle (Cₜ) values were calculated from the fluorescence signal ratio of the acceptor and donor fluorophores (detection channel F2/F1), using the second derivative maximum method. Using serial dilutions of the standard, we generated a calibration curve based on the linear relationship between the CT value and the logarithm of the starting copy number. Samples were quantified with the LightCycler analysis software, Ver. 3.5.

Results from a LightCycler run with serially diluted samples of standard RNA with detectable transcripts deriving from 35 (4.2 ag) to 3.5 × 10⁵ copies are shown in Fig. 1, A and B. The calibration curve (Fig. 1B) indicates a good correlation between the number of CYP3A4 mRNA copies and Cₜ (r = −0.98). The limit of quantification, because of loss of amplification linearity, was ~35 mRNA copies per assay (data not shown). Fig. 1C shows amplification of CYP3A4 mRNA from five leukocyte samples and three liver samples. In 31 healthy volunteers, CYP3A4 mRNA concentrations (mean of three determinations) ranged from 13 to 80 molecules/ng of total RNA. The mean was 34 molecules/ng of total RNA (SD = 13 molecules/ng of total RNA; n = 31). Three human liver samples containing high amounts of CYP3A4 mRNA were used as positive controls. A mean of 3 × 10⁵ molecules/ng of total RNA (5 × 10⁵, 1 × 10⁵, and 2 × 10⁵ molecules/ng of total RNA) was determined.

Whole blood (80 mL) was collected from one individual into 8-mL Vacutainer cpt tubes and divided into equal aliquots during lymphocyte preparation. For evaluation of the interday assay variation, CYP3A4 mRNA was isolated and measured on different days as described above, whereas for evaluation of the intraday assay variation CYP3A4 mRNA was isolated and measured on the same day. The interday CV for an assay with 40 ng of total RNA was 29% (n = 10), using single determinations with a measured CYP3A4 mRNA concentration of 53 copies/ng of total RNA; the corresponding intraday CV was 24% (n = 10) for a sample with 33 copies of CYP3A4 mRNA/ng of total RNA.

We evaluated the use of real-time quantitative RT-PCR technology for the detection of CYP3A4 gene expression in human liver samples and peripheral blood leukocytes by measurement of mRNA. Because the three well-described members of the CYP3A family (CYP3A4, CYP3A5, and CYP3A7) and the novel CYP3A43 have homologous sequences in common, specific primer selection remains the crucial step in RT-PCR design (1, 7, 8). Different oligonucleotides were tested (data not shown), but the

<table>
<thead>
<tr>
<th>Primer position*</th>
<th>Exonsb</th>
<th>Method</th>
<th>Quantification</th>
<th>Leukocytes</th>
<th>Liver</th>
<th>References</th>
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<tr>
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<td>Relative to β-actin</td>
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<tr>
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<td>Competitive to standard RNA</td>
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<td></td>
<td>LightCycler</td>
<td>Absolute</td>
<td>34 copies/ng</td>
<td>This study</td>
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* Primer location was defined according to sequence no. X12387 in the EMBL gene bank.

b Exon definition corresponds to sequence no. AF209389 in the EMBL gene bank.
² ND, not detected.
³ Number of cycles to obtain a product.
best results were provided by the chosen pair. Because the primers span intron 12, we also avoided the possibility of genomic DNA contamination.

Real-time RT-PCR is a rapid, specific, and sensitive method that allows the detection of a very low number of mRNA copies; the high sensitivity, with a detection limit of \( \sim 10 \) copies of cDNA, has already been reported \((9–11)\). However, even minor variations in reaction conditions, particularly during RNA extraction and reverse transcription, are multiplied by the PCR. The external RNA standard constructed for this assay could be used as a control for the efficiency of both RT-PCR steps because it is reverse-transcribed and amplified in parallel with native mRNA. Reaction conditions were identical for the CYP3A4 RNA standard and the native CYP3A4 mRNA because of sequence and primer similarity.

Assuming an average of 10–30 pg of total RNA in human cells (containing \( 0.2 \times 10^6 \) to \( 1 \times 10^6 \) molecules of mRNA), we found a mean of 0.5 copies of CYP3A4 mRNA per cell, which means that on average, only every second leukocyte expresses CYP3A4 mRNA. Another group \((6)\) has found a similar low CYP3A4 mRNA quantity in leukocytes. This marginal presence of CYP3A4 mRNA in peripheral blood lymphocytes might be explained by white cell differentiation. Indeed, Sempoux et al. \((5)\) reported that CYP3A proteins are expressed in B lymphocytes but not in T lymphocytes.

Several authors have tried to determine CYP3A4 mRNA concentrations in lymphocytes with different and partially contradictory results (Table I). Janardan et al. \((4)\) and Hukkanen et al. \((12)\), who used PCR methods in conventional thermocyclers and detection in agarose gels, did not find any CYP3A4 mRNA in lymphocytes, whereas Nakamoto et al. \((8)\) obtained detectable amounts of CYP3A4 in human lymphocytes in a relatively quantitative manner, using the housekeeping gene \( \beta \)-actin as a standard. However, because they chose both primers in the same exon, any possible contamination with genomic DNA may not have been differentiated from cDNA. Krovat et al. \((6)\) found marginal amounts of CYP3A4 mRNA in lymphocytes, using a quantitative competitive RT-PCR with conventional cycling, but the measured concentrations were at the detection limit of the assay. Recently, Westlind et al. \((13)\) discovered that no CYP3A4 mRNA could be detected in lymphocytes by the real-time TaqMan technology. We hypothesize that in this case, degradation of mRNA near the 5′ end could have taken place. In general, degradation of mRNAs can occur, depending on the respective specific sequences in the 5′-untranslated region, the coding sequence, and/or the 3′-untranslated region \((14)\). It is possible that primers that are placed near the 5′ end of the CYP3A4 mRNA cannot produce any amplicons because the mRNA may already be degraded in this area.

To correlate initial template concentration to real-time fluorescence curves, the LightCycler software offers two possibilities for a crossing-point determination. The first allows a definition of the fluorescence threshold, where the fluorescence signal exceeds the background. Because the detection limit of real-time instruments is \( \sim 100 \) copies, the threshold method assumes that all samples have the same, relatively high DNA concentration and requires manual definition of the exponential amplification phase. We decided to apply the second derivative maximum method, which assumes that the shape of the curve is a better guide to the concentration of the PCR product, and the crossing point should be at the maximum acceleration.

Methods for CYP3A4 quantification are of growing interest because many drug interactions can be traced back to an induction of CYP3A4 transcription. CYP3A4 is known to be induced by barbiturates, glucocorticoids, rifampicin, and many other drugs \((1–3)\), so that patients receiving multiple drug therapy could profit from the presented method. Conventional in vivo tests to measure CYP3A4 activity in humans use separately administered test substrates \((e.g., \text{alprazolam})\) and require their subsequent determination in blood or urine \((15)\). The real-time RT-PCR method described here represents an ex vivo assay that allows quick assessment of CYP3A4 in leukocytes.

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References
inborn errors of metabolism or congenital endocrine disorders have been greatly improved by the introduction of neonatal mass screening programs in many parts of the world. For this purpose, blood from newborns, ideally 4–7 days of age, is collected on filter-paper cards, dried, and mailed to a centralized laboratory, where various tests for congenital metabolic and endocrine disorders are performed. Until 2001 in Austria, several tests were done on blood spots on native cards as a first step; the cards were then autoclaved and screened for phenylketonuria, and the leftovers were stored at room temperature. In the Czech Republic, the cards are usually cut into two parts: one half is assayed native, whereas the other is autoclaved; leftovers from the native half are stored at room temperature. Since 2001, Austrian cards have been treated similarly. Accordingly, autoclaved cards are available for 1991–2000 from Austria, along with native cards, whereas from the Czech Republic, native cards are available dating back to 1988.

To evaluate the effect of autoclaving on 17-OHP concentrations in dried blood spots (experiment 1a), native filter-paper cards from 25 term and 25 premature neonates were retrieved from the Austrian neonatal screening bank. The cards were collected in 2001. The 17-OHP concentration in a blood spot on each native card was first assayed (native 17-OHP). Subsequently, the cards were autoclaved and the 17-OHP concentration was re-assayed (autoclaved 17-OHP).

To evaluate the effect of autoclaving on 17-OHP concentrations in a fluid environment (experiment 1b), an aqueous solution was prepared that contained 29.59 nmol/L 17-OHP (solution A). One-half of this solution was autoclaved in an open glass flask, and the evaporated fluid was substituted (solution B). Solutions A and B were dripped onto filter-paper cards, and 17-OHP was determined in six spots on each. Solution A was also dripped onto an additional filter-paper card, which was dried and autoclaved. The 17-OHP concentration in the autoclaved card was then determined (six replicates).

To evaluate the impact of long-term storage at room temperature on 17-OHP in autoclaved filter-paper cards (experiment 2a), we retrieved the first 40 cards from neonates fulfilling certain birth weight criteria (25 neonates weighing >2500 g, 5 weighing 2000–2500 g, 5 weighing 1500–2000 g, and 5 weighing <1500 g) from each year between 1991 and 2000 from the Austrian screening bank. A total of 400 cards were examined altogether.

To evaluate the impact of long-term storage at room temperature on 17-OHP in native filter-paper cards (experiment 2b), 520 cards from neonates born between 1988 and 2000 were retrieved from the Czech screening bank, according to the selection criteria described above (Fig. 1).