pre-purification by paper chromatography. The activity of the 11ß-HSD2 can be inhibited by glycyrrhetinic acid (GA), a constituent of liquorice. Administration of GA results in increased ratios of cortisol to cortisone in saliva, plasma and urine (7). In patients where liquorice-induced hypertension is suspected, screening for increased ratios of cortisol to cortisone in saliva might become a non-invasive and fast alternative for measurement of urinary GA (8).

Conclusion
We have developed a rapid, robust UPLC-MS/MS assay for the combined measurement of salivary cortisol and cortisone. This method can be used as a non-invasive and highly-specific tool to assess the value of salivary cortisol as a surrogate for free serum cortisol and as a potential novel way to assess 11ß-HSD2 activity, e.g. in studies on liquorice-induced hypertension.

References
adding two concentrations of 25(OH)D
replicates of four dilutions of 25(OH)D
albumin. Linearity was evaluated by measuring four
and 25(OH) D
respectively in serum samples containing 25(OH)D
limit of detection (LOD) and of quantification (LOQ)
according to the NCLS-EP10A3 protocol [3]. The
and high concentrations of 25(OH)D
prepared control serum samples with low, medium
fies more individuals (75.2%) with 25(OH)D concen-
trations above 50 nmol/l (table 1).

Discussion
 Several LC-MS/(MS) methods have recently been
described for the determination of 25(OH)D (2, 5),
with an inter-laboratory imprecision similar to most
immunoassays as based on results from laboratories
participating in DEQAS (6). How the assays are cali-
brated is a major factor to the LC-MS inter-laboratory
CV’s. Recently, it was demonstrated that LC-MS inter-
laboratory precision significantly improved after the
use of a common calibrator (7). We decided to cali-

Method validation
 We used commercial calibrator (160 nmol/l) and con-
trol samples (72.7 and 238 nmol/l) of human serum
origin (Chromsystems, Germany) and obtained intra-
assay variation from 14 replicate measurements in a
single series and inter-assay variation from 14 assays
over a 31 days period. In addition, intra-assay and to-
total imprecision were tested by analysis of three self-
prepared control serum samples with low, medium
and high concentrations of 25(OH)D (72.7, 117 and
205 nmol/l) and 25(OH)D (36, 117 and 205 nmol/l)
according to the NCLS-EP10A3 protocol [3]. The
limit of detection (LOD) and of quantification (LOQ)
was based on analyte signal/noise ratio of 3 and 10,
respectively in serum samples containing 25(OH)D
and 25(OH) D, after serial dilution in PBS with 60 g/L
albumin. Linearity was evaluated by measuring four
replicates of four dilutions of 25(OH)D and 25(OH)
D in PBS containing 60 g/l albumin in the range of
25-550 nmol/l (4). Analyte recovery was tested by
adding two concentrations of 25(OH)D (49.9 and
99.9 nmol/l) and 25(OH) D (54.3 and 108.6 nmol/l)
to three serum samples with 25(OH)D concentrations
ranging from 29.6-124.1 nmol/l, all with unmeasurable
basal 25(OH)D concentrations. Five samples from the
April 2009 distribution of DEQAS (an international
vitamin D external quality assessment scheme) were
analysed to determine the agreement of our LC-MS/
MS assay to other LC-MS participants (n=52). For
comparison we analysed 125 routine serum samples,
all with unmeasurable 25(OH)D concentrations,
with a manual 25-OH vitamin D 125I radioimmuno-
assay (DiaSorin) and a recently modified automated
electrochemiluminescent immunoassay (ECLIA) for
25(OH)D (Roche Diagnostics).

Results
 Intra-assay and total imprecision from NCLS-EP10
analysis were all below 8% for both 25(OH)D and
25(OH) D. For CS calibrator and control material, the
intra- and inter-assay imprecision were below 6.1%.
LOD was 1.5 nmol/l for 25(OH)D and 1.2 nmol/l for
25(OH)D. Respective quantification limits were 3.5
and 2.0 nmol/l. Both 25(OH)D and 25(OH) D were
linear to at least 550 nmol/l, with regression curves
y = 0.970x + 1.35 for 25(OH)D (r² = 0.9985) and y=
0.989x + 0.67 for 25(OH)D (r² = 0.9985). Observed er-
rors (0.39 nmol/l (3.9%) for 25(OH)D, and 0.63 nmol/l
(2.5%) for 25(OH)D) were within allowable systematic
error (0.4 (4%) and 1 (4%) nmol/l, respectively). The
mean recoveries were 99.5% (range 94.9-106.9%) for
25(OH)D and 95.4% (range 82.7-100.3%) for 25(OH)
D. Our LC-MS/MS results from DEQAS showed a
mean bias of -7.2%. Least-squares regression analysis
resulted in LC-MS external method mean = 1.01 x LC-
MS/MS + 4.40 (r² = 0.99)(n=5). Analysis of the CS
calibrator and control samples showed a bias of -11.5%
for 25(OH)D and -9.5% for 25(OH)D. The follow-
ing correlations from Deming regression analysis were
found: DiaSorin RIA = 0.975 (95% Confidence Interv-
al (CI): 0.919-1.031) x LC-MS/MS +3.02 (95% CI: 
-0.4-6.46); Sy/x= 8.01; r=0.90, and Roche ECLIA
= 0.948 (95% CI: 0.830-1.067) x LC-MS/MS + 13.01
(95% CI: 5.76-20.26); Sy/x= 16.90; r²=0.58 (figure
1A and B). The LC-MS/MS biased only 1.61 ± 8.11
nmol/l (bias ± SD) from the DiaSorin RIA, but 10.13 ±
17.31 nmol/l from ECLIA. When applying the cut-
off of 50 nmol/l for defining deficient versus normal
results, as proposed by Holick (1), the LC-MS/MS and
DiaSorin RIA classify 56.0% and 60.8%, respectively
as normal (>50 nmol/l) whereas Roche ECLIA classi-
fies more individuals (75.2%) with 25(OH)D concen-
trations above 50 nmol/l (table 1).
brate our LC-MS/MS assay on dilutions of pure standards of 25(OH)D_{3} and 25(OH) D_{2} in PBS containing albumin. This was preferred above the CS calibrator, as no details are given on how the CS assigned value was determined. When we measured the CS calibrator, as it was a patient sample, we found an approximate -10% deviation from target value for both 25(OH)D_{3} and 25(OH) D_{2}. In line with this are our results from DEQAS with a -7.2% bias of the LC-MS method mean. Depending on the number of laboratories participating in DEQAS using CS material for calibration, this might partly explain the negative bias of our LC-MS/MS to the LC-MS methods mean. The LC-MS/MS agreed well with the results obtained by using the DiaSorin RIA. The scatter around the regression curve between both methods is attributed to the relatively high imprecision (±10% CV) of the RIA, as judged from repeated measurements of some of the patient sera using both methods. Roche has recently

![Figure 1. Comparison of LC-MS/MS with manual radioimmunoassay (A, B) and automated immunoassay (C,D) in 125 patient serum samples. A and C: scatter plots; B and D: bias plots.](image)

<table>
<thead>
<tr>
<th>Assay method dependent accuracy in % of classification according to Holick (1) on the basis of ranges of 25(OH) D_{3} (n=125)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Severe Deficiency</td>
</tr>
<tr>
<td>&lt; 25 nmol/l</td>
</tr>
<tr>
<td>LC-MS/MS</td>
</tr>
<tr>
<td>RIA DiaSorin</td>
</tr>
<tr>
<td>ECLIA Roche</td>
</tr>
</tbody>
</table>

re-standardised their ECLIA assay to LC-MS/MS (8) giving approximately 10% lower values (Data from Roche Diagnostics). However, in comparison to our LC-MS/MS the ECLIA still overestimates 25(OH)D$_3$ concentrations up to 4-fold, particularly in the lower concentration range (<30 nmol/l). This might somehow be related to the limited sensitivity of the ECLIA having a LOD of 10 nmol/l. Also at higher concentrations (>75 nmol/l) large individual discrepant patient’s results were seen differing up to ± 50 nmol/l of 25(OH)D$_3$. Matrix effects distorting effective displacement of 25(OH)D$_3$ from its binding protein may be responsible for the large inter-method variability in some individual patient sera. Another possibility is cross-reaction with other vitamin D metabolites in the ECLIA. In conclusion, the described LC-MS/MS method provides a rapid, accurate and sensitive alternative to other methods for determination of 25(OH)D, with a real advantage being the ability to report separate results for 25(OH)D$_2$ and 25(OH)D$_3$. It compares well to the established DiaSorin radioimmunoassay but to a lesser extent to the recently re-standardised ECLIA vitamin D$_3$ assay from Roche.

Acknowledgements

We thank JPM Wielders, PhD (Meander Medical Center, Amersfoort, The Netherlands) for providing us with the DEQAS samples and Roche Diagnostics (Almere, The Netherlands) for the gift of kits free of charge for this study.

References