Rapid Quantification of Buprenorphine-Glucuronide and Norbuprenorphine-Glucuronide in Human Urine by LC–MS–MS

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Abstract

A liquid chromatography–tandem mass spectrometry (LC–MS–MS) method was developed and validated for the determination of buprenorphine-glucuronide (BUP-G) and norbuprenorphine-glucuronide (NBUP-G) in human urine. The method included a dilution step followed by filtration through a Mini-Uniprep Filter and direct injection onto the LC column. The analytes were quantified in multiple reactions monitoring mode using one transition ion. Norbuprenorpine-d_3 (NBUP-d_3) was used as the internal standard. The concentration ranges were 6–161 ng/mL for BUP-G and 12–295 ng/mL for NBUP-G. Recoveries determined after filtration for the analytes were 75%. The between-day precision of the method was in the range of 4.8–11%. The limits of quantification were found to be 4.6 ng/mL for BUP-G and 11.8 ng/mL for NBUP-G. Approximately 1000 samples from law enforcement, prison inmates, probation services, and hospitals were analyzed by the presented method. The ratios of drug glucuronides versus creatinine were calculated for a selection of samples (n = 151), where there was information on treatment with buprenorphine between 16 and 20 mg/day. The majority (86%) of the samples had a ratio of BUP-G/creatinine below 570 µg/g, and 76% of the samples had NBUP-G/creatinine lower than 1060 µg/g. The LC–MS–MS method proved to be robust and specific for the determination of BUP-G and NBUP-G in urine.

Introduction

Buprenorphine, which is a partial mu-opiate-receptor agonist and a kappa-opiate-receptor antagonist (1–3), has been used for many years in the treatment of opioid addiction in many countries. The efficacy of buprenorphine over placebo to reduce the use of opiates and the craving for opiates among opiate-addicted persons has been previously reported (4–7). Buprenorphine is mainly metabolized in the liver by *N*-dealkylation of its cyclopropyl group to form the metabolite norbuprenorphine (8,9). There is evidence that CYP 3A4 accounts for about 65% of norbuprenorphine production and CYP 2C8 accounts for about 30% (10–14). In urine, buprenorphine is predominantly excreted as glucuronide conjugates of buprenorphine and norbuprenorphine (9).

Several gas chromatography–mass spectrometry (GC–MS) studies for the analysis of buprenorphine and norbuprenorphine in urine have been presented (15–18). In GC–MS analysis, it is necessary to hydrolyze the urine specimens to release the analytes from their glucuronide conjugates followed by subsequent derivatization of the analytes to form thermostabile volatile compounds. During the derivatization step, buprenorphine and norbuprenorphine have been reported to undergo chemical rearrangement, and therefore, a reproducible derivatization may be difficult to obtain (19).

By using liquid chromatography (LC)–MS, direct analysis of the glucuronide conjugates is possible, and hence a simple pretreatment of the urine sample is permitted. The direct measurement of buprenorphine-glucuronide (BUP-G) and norbuprenorphine-glucuronide (NBUP-G) in plasma using LC–MS–MS have been published (20–22). A screening LC–MS–MS method for the determination of BUP-G and NBUP-G in urine has been published (23). The reference materials of the glucuronide metabolites were isolated from urine by preparative LC, and the measurement of conjugates was performed by LC–MS–MS. Standard reference solutions of BUP-G and NBUP-G have become commercially available and make quantification of the glucuronides easier. A quantification method for BUP-G and NBUP-G in urine using solid-phase extraction (SPE) followed by LC–MS–MS was recently published (21).

During the last year, this laboratory has received an increasing number of urine samples for buprenorphine analysis. The aim of the present study was to develop a simple, robust, and specific LC–MS–MS method for the simultaneous quantification of BUP-G and NBUP-G in urine that was suitable for routine analysis of forensic samples. Such a method would increase the efficiency of our routine by replacing a GC–MS method that requires enzymatic hydrolysis, SPE extraction, and derivatization prior to instrumental analysis.

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Material and Methods

Chemicals and reagents

BUP-G and NBUP-G were obtained from Cerilliant (Austin, TX) and ElSohly Laboratories (Oxford, MS). Norbuprenorphine-d_3 was purchased from Cerilliant. Other chemicals were of high-performance liquid chromatography (HPLC) or analytical grade from various commercial sources. The Mini-UniPrep-filters (0.2 nmol/mL PTFE) were purchased from Whatman (Clifton, NJ).

Standard solutions

For BUP-G, two separate stock solutions were prepared to a concentration of 16.0 and 21.2 µg/mL in methanol and were used as calibrator and control, respectively. The corresponding solutions of NBUP-G contained 19.0 and 24.6 µg/mL, respectively. Standard and control solutions were prepared by appropriate dilutions of a stock solution with water. Calibration solutions were prepared in blank urine at concentrations of 6.4, 12.9, 32.2, 64.4, and 160.9 ng/mL for BUP-G and 11.8, 23.6, 59.0, 117.9, and 294.8 ng/mL for NBUP-G. Quality control (QC) samples for BUP-G and NBUP-G were prepared in blank urine at concentrations of 9.7 and 51.5 ng/mL and 17.1 and 94.3 ng/mL, respectively. Calibration solutions and QCs were prepared before each analysis. The internal standard (IS) solution was diluted with acetonitrile/water (1:1, v/v) to a concentration of 54.1 ng/mL.

Sample treatment

Urine samples (0.25 mL) were diluted with 0.25 mL IS solution in acetonitrile/water (1:1, v/v) and filtered through 0.2-µm Mini-UniPrep Filter vials using a Mini-UniPrep six position compressor (Whatman).

Instruments

HPLC. LC was performed using a Waters Alliance 2695 system (Manchester, U.K.). Separation was performed on a Waters Symmetry C18 (2.1 * 100 mm, 3.5 µm) column, using gradient elution at a flow rate of 0.3 mL/min with the following solvent system: 100% acetonitrile (A) and 5mM ammonium acetate (pH 5) (B). The system was run in a linear gradient from 10% A to 30% A for 5 min and increased up to 80% A for 4 min. Re-equilibration of the HPLC column was achieved as the start conditions were held for 3 min before the next injection. The column temperature was held at 35°C during analysis. The injection volume was 10 µL.

MS–MS. A Quattro Premier tandem-quadrupole MS (Waters) equipped with a Z-spray electrospray interface was used. Positive ionization was performed in the multiple reaction monitoring (MRM) mode. The capillary voltage was set to 1.0 kV, the source block temperature was 120°C, and the desolvation gas (nitrogen) was heated to 400°C and delivered at a flow rate of 890 L/h. The cone gas (nitrogen) was set to 60 L/h, and the collision gas (argon) pressure was maintained at 0.5 psi. The *m/z* 644.1 → *m/z* 468.1 transition (cone voltages, 65 V; collision energy, 40 eV) was monitored for BUP-G, *m/z* 590.1 → *m/z* 414.2 transition (cone voltages, 65 V; collision energy, 35 eV) for NBUP-G and *m/z* 417.1 → *m/z* 101.1 transition (cone voltages, 70 V; collision energy, 40 eV) for norbuprenorpine-d_3 (NBUP-d_3). System operation and data acquisition were controlled using Mass Lynx 4.0 software (Waters). All data were processed with the QuanLynx quantification program (Waters).

Identification and quantification

The analyte specimens were identified by comparing their retention times with the retention times of the corresponding standards and control samples, and the retention times should not deviate more than ± 2% from the average ratio. The appearances of both analytes were also used to confirm the intake of buprenorphine. Cases were reported negative in samples positive for NBUP-G only, with no presence of BUP-G.

Peak-height ratios of analytes and internal standard of the calibration standard were used to generate a weighted (1/*x*) linear regression curve and applied to the peak-height ratios of calibrators, QCs, and unknown samples. The following was used to convert from nanograms per milliliter to nanomoles: nanograms per milliliter/molecular weight × 1000. The molecular weight for BUP-G was 643.7, and for NBUP-G, it was 589.6.

Method validation

The extraction recovery for the filtration method was determined with six replicates at 12.9 ng/mL for BUP-G and 23.6 ng/mL for NBUP-G. Recoveries were estimated by comparing peak heights obtained when the analytes were added before sample preparation with those obtained when the analytes were added after the filtration step. The internal standard was added after filtration in both cases. Within-day precision and accuracy was estimated by analysis of 10 separate preparations of the lowest QC concentration level (BUP-G, 9.7 ng/mL and NBUP-G, 17.7 ng/mL) in a single assay. Between-day precision and accuracy were determined by anal-

Table I. Concentration Range of Calibration Curves and LOD and LOQ (n = 10)

Analyte	Concentration (ng/mL)	LOD (ng/mL)	LOQ (ng/mL)
BUP-G	6.4–160.9	1.4	4.6
NBUP-G	11.8–294.8	3.8	11.8

Table II. Between-Day Precision and Accuracy (n = 10) of the Analytes

Analyte	Added (ng/mL)	Mean Concentration Found (ng/mL)	RSD (%)	Accuracy (%)
BUP-G	9.3	9.9	4.8	6.5
	50.8	53.7	6.0	5.7
NBUP-G	17.3	19.2	11.0	11.0
	94.4	108.7	8.2	15.1

ysis of aliquots of each QC concentration at 10 different days over a 3-month period. Limits of detection (LOD) and quantification (LOQ) were determined by analyzing five different drug negative urine specimens on 10 successive days, one replicate on each day. Drug-negative urine specimens (2 replicates on 10 different days) spiked to a concentration near the presumed LOD, with a signal-to-noise ratio of at least 10 (1.9 ng/mL BUP-G and 3.5 ng/mL NBUP-G) were analyzed. LOD and LOQ were defined as the analyte concentration giving a MRM transition equal to that of the mean of the negative samples plus 3 and 10 standard deviations of the LOD sample, respectively. The analyses of approximately 180 samples were compared with the previous routinely used GC–MS method (24).

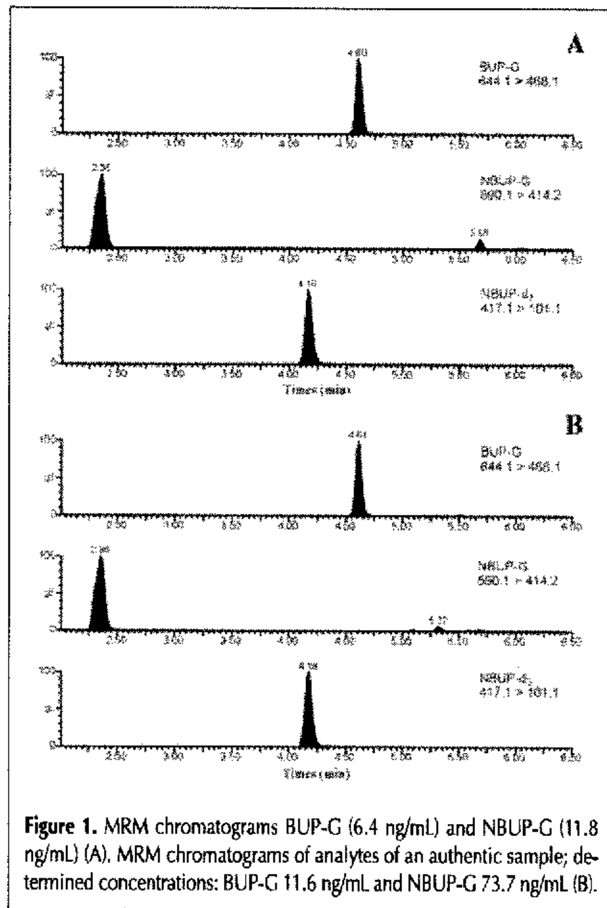

Figure 1. MRM chromatograms BUP-G (6.4 ng/mL) and NBUP-G (11.8 ng/mL) (A). MRM chromatograms of analytes of an authentic sample; determined concentrations: BUP-G 11.6 ng/mL and NBUP-G 73.7 ng/mL (B).

Forensic samples

Urine samples were sent to our laboratory from law enforcement, prisons, probation services, and hospitals for control or misuse of treatment. In the vast majority of our cases, we are asked to confirm the use of buprenorphine as a part of medical treatment for opioid addiction or to verify illegal use of the buprenorphine. In some cases, the measured concentration of analytes needed to be used to interpret whether there was a new illegal intake or trace the old illegal intake, especially in prisoners without information on legal use. The specimens were stored at 4°C until analyzed (maximum of 1 week). Buprenorphine was analyzed as it was requested in approximately 1000 samples. Information on buprenorphine treatment was present, with doses varying between 16 and 20 mg/day, for 151 urine samples. Screening of urine samples was performed with cloned enzyme donor immunoassay (CEDIA) buprenorphine assay (Microgenics GmbH, Passau, Germany). Positive results were confirmed by LC–MS–MS. The urine creatinine concentration was analyzed using the Jaffé method (Roche Diagnostics GmbH, Mannheim, Germany).

Matrix effects

For the investigation of matrix effects the method described by Matuszewski et al. (25) was used. Set 1 consisted of neat calibration solutions and set 2 were extracts of six different urine specimens spiked (after the filtration) with the same amount of calibration solutions used for set 1. The mean peak heights and relative standard deviations (RSDs) of set 1 and set 2 were calculated. The matrix effect (ME %) was determined by equation

Table III. Matrix Effect

		Set 1* (n = 5)		Set 2† (n = 6)		Set 2† (n = 6)		
Analyte	Added (ng/mL)	Mean Peak Height‡	RSD %	Mean Peak Height‡	RSD %	Mean Peak Ratio Analyte/ISTD	RSD %	ME %§
BUP-G	12.9	3.9	2.2	9.8	24.3	5.4	22.5	253
	64.4	19.6	3.2	45.8	33.0	11.9	23.3	234
NBUP-G	23.6	3.1	4.0	2.7	13.2	1.7	28.1	85
	117.9	15.2	1.0	13.2	13.0	3.7	26.6	87
NBUP-d_3	8.3	1.2	2.8	1.7	14.4			144
	41.7	2.6	2.8	3.9	18.4			152

* Neat standards.
† Compounds spiked after extraction into extracts from six different urine specimens.
‡ In arbitrary units, × 10^4.
§ Matrix effect expressed as the ratio of the mean peak height of a compound spiked after filtration (set 2) to the mean peak height of the same compound standards (set 1) multiplied by 100. A value of > 100% indicates ionization enhancement, and a value of < 100% indicates ionization suppression.

1. ME values of 100% indicated the absence of any matrix effects, values < 100% indicated ion suppression, and values > 100% indicated ion enhancement.

$$\text{ME }\% = \frac{\text{Mean peak height Set 2}}{\text{Mean peak height Set 1}} \times 100 \qquad \text{Eq. 1}$$

A post-column infusion system was used for the control of possible ion suppression (26). Four separate stock solutions containing the analytes (BUP-G, 45.1/450.6 ng/mL and NBUP-G, 41.4/412.7 ng/mL) were prepared in acetonitrile/water (50:50), which was infused post-column using a syringe pump (10 µL/min) directly into a tee-coupling and mixed with mobile phase. Aliquots of 10 µL of blank filtered urine were injected into the HPLC.

Specificity

In order to investigate the specificity of the method, several opiates/opioids, cocaine, and benzoylecgonine [morphine-glucuronide, morphine, codeine, folkodine, 6-acetylcodeine, 6-monoacetylmorphine (6-MAM), oxycodone, ethylmorphine, dihydrocodeine, norcodeine, and methadone] at concentrations approximately 750 ng/mL were injected into the LC–MS–MS system. Real samples known to contain high concentrations of codeine and ethylmorphine and their respective glucuronides were analyzed. A mixture of 6-MAM, morphine, and codeine (81.85, 71.33, and 74.83 µg/mL) was added to a low QC sample. The sample pre-treatment was performed as described for the LC–MS–MS method.

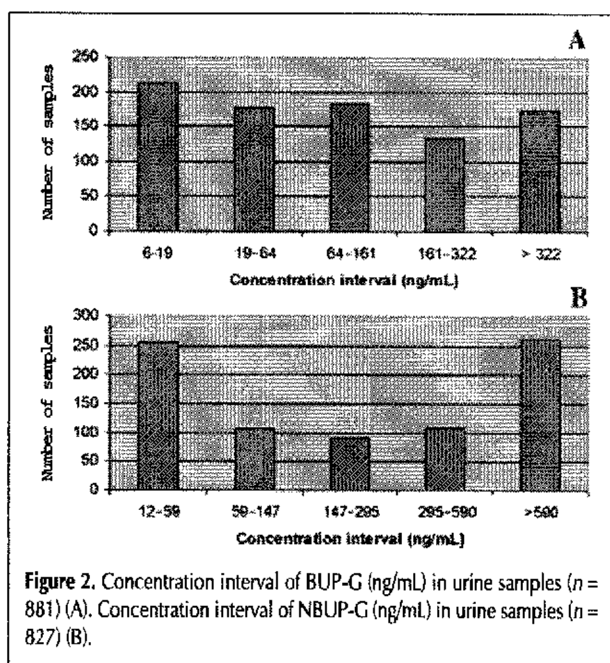

Figure 2. Concentration interval of BUP-G (ng/mL) in urine samples (n = 881) (A). Concentration interval of NBUP-G (ng/mL) in urine samples (n = 827) (B).

Results and Discussion

A selective SPE method for glucuronides was difficult to obtain because of the water-solubility of the compounds. SPE extraction was evaluated during method development. However, the sample cleanup was not satisfactory, and a simple, time-saving dilution and filtration method was therefore developed. Because of unfavorable fragmentation of buprenorphine and norbuprenorphine, several previous studies (23,27,28) probably measured the transfer of the intact molecular ion to the second analyzer. Our dilution and filtration method allowed for the more selective detection of the specific product ions of BUP-G and NBUP-G in MS–MS analysis. With the gradient conditions chosen, NBUP-d_3 was eluted close to BUP-G, whereas buprenorphine-d_4 was eluted in the end of the gradient run. NBUP-d_3 was therefore used as internal standard for BUP-G and NBUP-G because a deuterated analogue of BUP-G was not commercially available.

Method validation

Calibration curves were made for each compound for the concentration range listed in Table I. The calibration curves were found to be reproducible within the concentration range listed.

The linearity of the calibration curve was also tested up to 321.8 ng/mL for BUP-G and 589.6 ng/mL for NBUP-G. Both curves proved to be linear. In those cases where it was important to measure the exact concentration, the samples were diluted and reanalyzed. The LOQs for BUP-G and NBUP-G were calculated to be 4.5 and 11.8 ng/mL, respectively (Table I). The between-day precision and accuracy of the analytes are presented in Table II. The RSDs of both analytes ranged between 4.8% and 11.0%. The within-day variation (RSD) was between 3.5% and 4.6%, and the accuracies of BUP-G and NBUP-G were 1.0% and 9.7%, respectively (data not shown). Recoveries of both analytes, after filtration, were 75% with RSD values up to 14%. Figures 1A and 1B present MRM chromatograms of the lowest concentration calibrator and an authentic urine specimen, respectively.

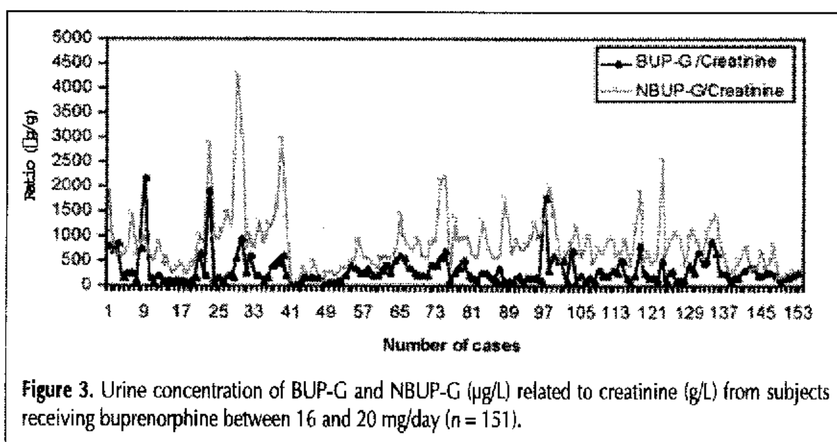

Figure 3. Urine concentration of BUP-G and NBUP-G (µg/L) related to creatinine (g/L) from subjects receiving buprenorphine between 16 and 20 mg/day (n = 151).

Comparison of LC–MS–MS with GC–MS

During the validation period, approximately 180 samples were analyzed using both the new LC–MS–MS method and the previously used routine GC–MS method. Samples that gave a positive result by the GC–MS method were also positive by the LC–MS–MS method. Some of the samples (3%) were unsuitable for the GC–MS method. In these samples, BUP-G and NBUP-G were successfully measured by the new method.

Matrix effect

The extent of matrix effects was tested by analyzing filtered blank urine fortified with analytes and internal standard at two concentrations. The peak heights were compared to directly injected standards at the same concentrations. The results are presented in Table III. The signals of BUP-G and NBUP-d_3 in spiked matrix were increased compared to the neat standard, whereas the signal in set 2 for NBUP-G was slightly decreased. The higher variability in set 2 than in set 1 indicated a matrix effect. The RSDs for set 2 using the peak-height ratio of drug and internal standard showed improved RSDs for BUP-G when compared to no correction with internal standard. This indicated that the internal standard had a compensatory effect both on the precision and reliability of the quantification of BUP-G. However, the correction with internal standard had an opposite effect for NBUP-G, whereas the RSDs were increased. The matrix effect was further investigated using post-column infusion of analytes. However, the post-column infusion experiment did not show any matrix suppression for injection of blank pretreated matrix.

The use of deuterated internal standard can partly overcome the problem of matrix effects. Therefore, deuterated analogues of BUP-G and NBUP-G will be evaluated with the present method when they are commercially available. During the months the method has been used for routine analysis, and calibrators and control samples have proven to be stable and reproducible. Thus, the observed matrix effects were considered to be acceptable.

Specificity

The method specificity was demonstrated by the absence of interfering substances co-eluting with the analytes in 10 different lots of negative urine specimens. Several opiates/opioids, cocaine, and benzoylecgonine were injected into the LC–MS–MS system. In addition, samples containing high concentrations of ethylmorphine-glucuronide and codeine-glucuronide were also analyzed. None of the compounds co-eluted with either NBUP-G or BUP-G. Furthermore, the addition of a mixture of opiates to a low QC sample did not affect the quantification of the analytes.

Application

Approximately 97% of the CEDIA screening positive samples were confirmed by the presented method. Of the 928 samples confirmed by the LC–MS–MS method, 881 were positive for BUP-G and 827 for NBUP-G. In most cases, a combined presence of both compounds was observed, but in some samples only one of the analytes was present above the lowest calibration standard. Thus, BUP-G had to be above our cut-off value in order to consider the sample positive for buprenorphine intake, knowing that the sample was also positive for CEDIA screening test. The distributions of BUP-G and NBUP-G urine in samples from law enforcement, prison and probation services, and hospitals are presented in Figures 2A and 2B. Concentration levels below 160.9 ng/mL (the highest calibrator standard of BUP-G) were found in 65% ($n = 573$) of the samples positive for BUP-G. In 55% ($n = 455$) of the NBUP-G positive samples, the concentrations were below the highest calibrator at 294.8 ng/mL.

In a few of the urine specimens ($n = 4$) a shift in the retention time was observed for the BUP-G and internal standard (approximately 1 min). The pH in those samples were found to be high (pH > 8). These samples were reanalyzed and the pH was adjusted to 5–6 with acetic acid before dilution and filtration, resulting in expected retention times.

In a selection of urine samples where there was information of receiving buprenorphine for treatment of opioid addiction ($n = 151$), the ratios of BUP-G/creatinine and NBUP-G/creatinine were calculated (Figure 3). The majority (86%) of the samples had BUP-G/creatinine below approximately 570 µg/g, and 76% of the samples had NBUP-G/creatinine under approximately 1060 µg/g. The median ratio of BUP-G/creatinine to NBUP-G/creatinine was 3 (range 0–21). There is currently very limited information available about the ratio of BUP-G/creatinine and NBUP-G/creatinine in urine. In addition, we cannot be completely certain that the information regarding use of buprenorphine between 16 and 20 mg/day is correct (implies intake). However, these results could be useful in interpreting urine results with respect to monitoring treatment or to investigate drug exposure, although caution is advised in generalizing from our BUP-G/creatinine and NBUP-G/creatinine results.

Conclusions

A rapid and specific dilution and filtration method for the analysis of BUP-G and NBUP-G by LC–MS–MS in urine has been developed. This method greatly reduced the amount of time compared to the GC–MS method that required enzymatic hydrolysis, SPE extraction, and derivatization prior to instrumental analysis. The method has shown to be simple and robust in routine use during the past 8 months when approximately 1000 samples have been analyzed.

Acknowledgments

The authors would like to thank Jean-Paul Bernard and Jørg Mørland for critical reading of the manuscript and useful comments and Minna Hannele Ahtorinne for data collection and preparation.

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Manuscript received September 29, 2006;
revision received January 4, 2007.