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Validated semiquantitative/quantitative screening of 51 drugs in whole blood as silylated derivatives by gas chromatography-selected ion monitoring mass spectrometry and gas chromatography electron capture detection

Teemu Gunnar*, Sirpa Mykkänen, Kari Ariniemi, Pirjo Lillsunde

Laboratory of Substance Abuse, National Public Health Institute, Mannerheimintie 166, Helsinki 00300, Finland

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Abstract

A comprehensively validated procedure is presented for simultaneous semiquantitative/quantitative screening of 51 drugs of abuse or drugs potentially hazardous for traffic safety in serum, plasma or whole blood. Benzodiazepines (12), cannabinoids (3), opioids (8), cocaine, antidepressants (13), antipsychotics (5) and antiepileptics (2) as well as zolpidem, zaleplon, zopiclone, meprobamate, carisoprodol, tizanidine and orphenadrine and internal standard flurazepam, were isolated by high-yield liquid-liquid extraction (LLE). The dried extracts were derivatized by two-step silylation and analyzed by the combination of two different gas chromatographic (GC) separations with both electron capture detection (ECD) and mass spectrometry (MS) operating in a selected ion-monitoring (SIM) mode. Quantitative or semiquantitative results were obtained for each substance based on four-point calibration. In the validation tests, accuracy, reproducibility, linearity, limit of detection (LOD) and limit of quantitation (LOQ), selectivity, as well as extraction efficiency and stability of standard stock solutions were tested, and derivatization was optimized in detail. Intra- and inter-day precisions were within 2.5–21.8 and 6.0–22.5%, and square of correlation coefficients of linearity ranged from 0.9896 to 0.9999. The limit of quantitation (LOQ) varied from 2 to 2000 ng/ml due to a variety of the relevant concentrations of the analyzed substances in blood. The method is feasible for highly sensitive, reliable and possibly routinely performed clinical and forensic toxicological analyses.

Keywords: Screening; Derivatization, GC; Drugs of abuse

1. Introduction

Substance abuse is an increasing societal problem throughout the world. From the point of view of toxicological laboratories, one of the key tasks is to identify the existence of these compounds in intoxicated drivers using whole blood as a biological matrix. It is a part of the process of continuously improving traffic safety.

Numerous drugs lower psychomotor skills, reaction time and observational capabilities, and therefore decrease the driving ability. Illicit drugs, such as opiates, cocaine, cannabinoids, amphetamine and its derivatives, in addition to therapeutic drugs such as benzodiazepines, antidepressants, antiepileptics and antipsychotics, are of primary concern in forensic toxicology due to their widespread occurrence and potentially hazardous effects in traffic. These drug classes have been shown to have severe, unexpected or unknown effects on psychomotor performance and traffic safety in clinical tests and simulated driving experiments [1].

In general, urine is used as a primary specimen in forensic screening analyses, owing to the higher concentrations and longer detection window of compounds of interest, compared to whole blood. Nevertheless, acute toxicity as well as the impairing effects of drugs on driving ability correlate with the concentration levels of substances that exist in blood, not in urine. Furthermore, in countries like Finland, Sweden, Germany and Belgium, a so-called ‘zero tolerance law’ is applied to illicit drugs or drugs under international

* Corresponding author. Fax: +358-9-47448553.
E-mail address: teemu.gunnar@ktl.fi (T. Gunnar).

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control, if these drugs or their active metabolites are found in the blood of drivers. Therefore, blood is the most important specimen in 'drugs and driving' cases.

A complex whole blood matrix presents a challenge to analytical screening procedures, as they have to be simultaneously rapid, sensitive and reliable, and only a small sample must result in highly comprehensive identification power, and possibly simultaneous quantitation, of forensically significant compounds. In drug analyses on whole blood and serum, immunoassay techniques are widely used for screening, due to their rapidity and absence of sample handling [2–8]. They nevertheless have serious limitations in sensitivity and selectivity, and may cause both false negative and, especially, false positive results. Consequently, chromatographic solutions have been developed to increase the reliability of the screening procedures. Recently, two chromatographic separation techniques utilizing nitrogen phosphorus detector (NPD) were reported [9,10]. Prior to this method, we used the combination of NPD and electron capture detector (ECD) in a dual-column system in our laboratory [2]. Further improvements in reliability can be achieved by hyphenated chromatographic techniques, i.e. gas chromatography–mass spectrometry (GC–MS) [11–15], and liquid chromatography–mass spectrometry (LC–MS) [16–18]. The former is still preferred in toxicological analyses owing to its better sensitivity with several compounds, lower cost of analysis and manifold acquisition costs of LC–MS system.

A large majority of the GC–MS blood screening methods operate in a full-scanning mode and are based on mass spectral libraries and search algorithms. Apart from continuous improvements in that area, library search still has its limitations [19]. Furthermore, the screening procedures based on scanning mode do not usually have sufficient sensitivity to identify all significant compounds on the total ion chromatogram (TIC). Improved sensitivity facilitates, e.g. analyses of the low-dosed benzodiazepines and cannibinoids in a selected ion-monitoring (SIM) mode. We, therefore, approached the screening problem in a way, which resembles confirmatory analysis, by choosing first the compounds of interest and analyzing them in SIM mode. The loss of compound specific TIC was not considered to be problematic, because the identification was based on accurate retention time, the most intensive compound specific ions and their relative ion abundances like in the state-of-the-art confirmatory GC–MS analyses.

The majority of substances are detected after *tert*-butyldimethylsilylation (MTBSTFA) and trimethylsilylation (MSTFA) by GC–SIM–MS. Zaleplon, zopiclone and various low-dosed benzodiazepines are analyzed in their underivatized forms prior to the derivatization procedure by GC–ECD, due to their lack of sensitivity at therapeutic concentrations in mass spectrometric detection using electron impact (EI) ionization. Furthermore, combining two detection techniques in the same analysis allows double-screening of benzodiazepines with two detectors in one method.

2. Experimental

2.1. Chemicals, reagents and materials

Alprazolam and 6-monoacetyl morphine were donated by United Nations Narcotics Laboratory (Vienna, Austria). Dextropropoxyphene and metadone hydrochlorides, codeine phosphate, and pholcodine were from Leiras (Turku, Finland), as well as chlorpromazine, doxepin, ethyl morphine, fluoxetine, thioridazine and tramadol hydrochlorides, carbamazepine, chlordiazepoxide, diazepam, levomepromazine, meprobamate, nitrazepam, oxazepam, temazepam were from Orion Corporation (Espoo, Finland). Flurazepam was a donation from National Agency for Medicines (Helsinki, Finland) and phenazepam from the Republican Centre of Forensic Medicine (Moscow, Russia). Fluvoxamine maleate was from Solvay Pharmaceuticals (CP Weesp, The Netherlands). Chlomipramine and tizanidine hydrochlorides were purchased from Novartis (Basel, Switzerland). Imipramine and 10-monohydroxycarbamazepine were acquired from Ciba-Geigy (Basel, Switzerland). Clonazepam, midazolam and nordazepam were obtained from Roche (Mannheim, Germany). Amitriptyline was purchased from Star (Tampere, Finland), carisoprodol from Medipolar (Oulu, Finland), and orphenadrine from Lääke (Turku, Finland). Chlorprotixene and nortriptyline hydrochlorides, and citalopram hydrobromide were from H. Lundbeck (Copenhagen, Denmark). Cocaine hydrochloride was purchased from Sigma (St. Louis, MO, USA). Lorazepam, promazine and venlafaxine hydrochlorides as well as zaleplon were obtained from Wyeth-Ayerst Laboratories (Pearl River, NY, USA). Mianserin and mirtazapine were from Organon (Roseland, NJ, USA). Morphinesulfate pentahydrate was obtained from RBI (Natick, MA, USA). Sertraline hydrochloride was acquired from Pfizer (Cork, Ireland). Triazolam was from Pharmacia & Upjohn (Kalamazoo, MI, USA) and zolpidem hemitartrate from Sanofi-Synthelabo (Paris, France). Trimipramine maleate was from Aventis (Frankfurt, Germany) and zopiclone from Rhone-Poulenc (Courbevoie, France). Ampoules of THC (1 ng/ml), THC-OH (100 μg/ml) and THCC (100 μg/ml) were purchased from Radian Corporation (Austin, TX, USA).

Silylating reagents, *N*-methyl-*N*-(trimethylsilyl)trifluoroacetamide (MSTFA) and *N*-methyl-*N*-(*tert*-butyldimethylsilyl)trifluoroacetamide (MTBSTFA) were acquired from Aldrich (Milwaukee, WI, USA). Butyl acetate, methanol, acetonitrile and Na_2HPO_4 of analytical grade, were supplied by Merck (Darmstadt, Germany). Adult bovine serum was purchased from Biological Industries (Kibbutz Beit Haemek, Israel).

2.2. Sample treatment

Whole blood sample, serum standard, control or blank (1 ml), 0.5 M Na_2HPO_4 (1 ml) and butyl acetate (5 ml) including flurazepam (200 ng/ml) in extraction solvent as

internal standard, were added to a disposable 15 ml glass tube. The mixture was strongly vortexed for 30 s followed by centrifugation (1700 × *g*, 5 min). The organic layer was transferred to a similar test tube and evaporated to dryness under a stream of air in a water bed at 75 °C (15–20 min). To the dry extract, 100 μl of acetonitrile was quickly added prior to sample dividing. An aliquot of 30 μl was transferred to 200 μl inserts, sample vials were tightly capped, and the substances were identified in their underivatized forms by gas chromatography combined with electron capture detection. The rest of the sample (70 μl) was derivatized by 15 μl of MTBSTFA and heated (80 °C, 30 min) in capped test tubes to complete the optimized derivatization reaction. After significant cooling to the ambient temperature (10 min), 15 μl of MSTFA were added to the samples and the compounds were analyzed in their underivatized, *tert*-butyldimethylsilylated (TBDMS) and trimethylsilylated forms (TMS) by gas chromatography–mass spectrometry.

2.3. Gas chromatography

All gas chromatographic separations connected to electron capture detection were performed with a Hewlett-Packard 5890 Series II gas chromatograph (Palo Alto, CA, USA) equipped with a 30 m DB-35 (0.53 mm i.d., 1.0 μm film thickness) fused silica capillary column from J&W scientific (Folsom, CA, USA). The oven temperature was initially maintained at 250 °C for 2 min, then raised at a rate of 15 °C per min to 320 °C, and held for 5 min. Helium 4.6 (99.996%) was used as the carrier gas and argon–methane as the make-up gas with a constant flow of 5.8 and 30 ml/min, respectively. The injector and detector temperatures were maintained at 280 and 300 °C. Aliquots of 2.0 μl were injected into the GC using a split ratio 7:1.

2.4. Gas chromatography–mass spectrometry

A Hewlett Packard 6890 Gas Chromatograph was interfaced to a HP 5973 Mass Selective Detector (Palo Alto, CA, USA). Gas chromatographic separations were performed using a cross-linked 30 m DB–35 ms silica capillary column (0.32 mm i.d., 0.25 μm film thickness) from J&W scientific (Folsom, CA, USA). The operation conditions were as follows: the initial temperature was 120 °C for 1 in, then raised at a rate of 15 °C per min to 320 °C, and held constant for 4 min. Solvent delay was set at 4 min and helium 5.6 (99.9996%) was used as the carrier gas at a constant flow of 1.5 ml/min. A HP split/splitless injector was used in the splitless mode, and aliquots of 2.0 μl were introduced to the chromatographic system with an injection pressure of 34.27 kPa up to 1.0 min. The injection port, transfer line, quadrupole and ion source temperatures were set at 250, 300, 150 and 230 °C, respectively.

In the mass spectrometric measurements, the electron impact mode was used at 70 eV. The scanning range at the full scan mode was set to 50–550 Da to identify unknown ions and their relative abundance of the separated compounds of interest in EI. The developed method was carried out in SIM mode. Mass spectrometer tuning was performed weekly by optimized target tuning macro to emphasize the intensities of larger ion masses. The applied values are given in Table 1. The condition of the mass spectrometry was checked daily by autotuning, using heptafluorotributylamine (HFTBA). Data handling and system operations were controlled by HP Chemstation software (B.02.05).

Table 1
Target tune parameters

Tune targets (*m/z* 69 = 100%)
Mass 50 target (0.3–5.0%): 1%
Mass 131 target (20–120%): 55%
Mass 219 target (20–120%): 120%
Mass 414 target (0.3–10%): 10%
Mass 502 target (0.3–10%): 10%

2.5. Samples, standard solutions and validation

Throughout the study, whole blood samples taken from drivers suspected of intoxication, were used. Commercial bovine serum was used in the validation tests. Validation tests were performed in significant concentration ranges of the examined substances based on both experience and literature [1,20]. In addition, larger concentrations were studied in linearity tests to evaluate the performance of the method in overdose cases.

2.5.1. Linearity and limit of detection

Standard solution of a certain drug was prepared in methanol or acetonitrile at a concentration of 1.0 and 10.0 mg/ml (carisoprodol and meprobamate) of free substance or their salt. Cannabinoids were readily purchased in methanolic media (ampoules) at a concentration of 1.0 mg/ml (THC) and 0.1 mg/ml (11-OH-THC, THCC). Standard solutions were kept at +4 °C. Compounds were divided in to two working standard solutions using the above mentioned standard solutions (10.0, 1.0, 0.1 mg/ml) and diluted in bovine serum based on the typical concentration levels of the analyzed substances. Working standard solution A contained 31 compounds, such as the low-dosed benzodiazepines (triazolam, clonazepam, nitrazepam, lorazepam, midazolam and alprazolam), cannabinoids (THC, 11-OH-THC, THCC) and opioids (morphine, 6-monoacetyl morphine, ethyl morphine, codeine, tramadol, pholcodine and methadone). Working standard solution B contained 20 compounds, e.g. the rest of the analyzed benzodiazepines (diazepam, nordazepam, oxazepam, chlordiazepoxide, phenazepam and temazepam). GC–MS selected ion chromatograms of the spiked serum standard solutions A and B are shown in Figs. 1 and 2. The solutions were used in linearity, accuracy and interday precision tests.

The concentration range used for linearity experiment for each substance is informed in Table 2. Nine different

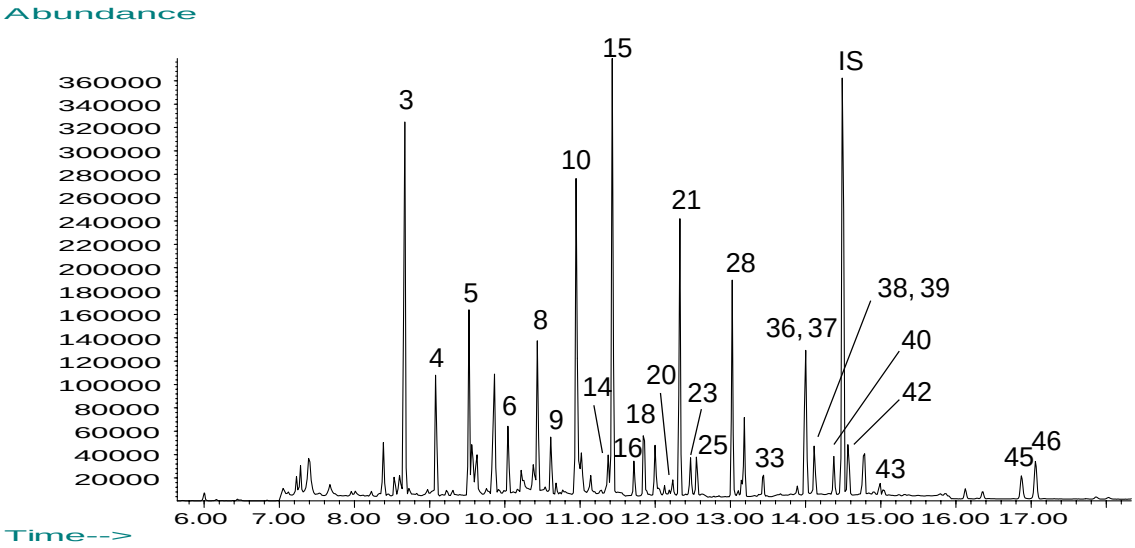

Fig. 1. GC–MS selected ion chromatogram of the spiked serum standard solution A. The concentration levels of the derivatized compounds are the same as the highest standard. For the peak identification, the numbers refer to Table 3. Imipramine is not included. For the instrumental information and temperature program, see Section 2.4.

concentration levels (1 replicate) of each substance were used. For example, the concentration points for tramadol were 5000 (working standard solution A_1), 2500, 1000, 500, 250, 100, 50, 25, 10 ng/ml and for meprobamate 25,000 (working standard solution B_1), 12,500, 5000, 2500, 1250, 500, 125, 50 ng/ml. The concentration points for other substances are equal or different (depending on the therapeutic concentration), but always relatively comparable to each other. All dilutions from working standard solutions A and B were made in bovine serum. The regression line was formed using least squares regression model. The relative response between compound and IS was accepted linear, if the square of correlation coefficient (R^2) exceeded 0.985. The limit of detection (LOD) of the analyzed drugs were determined from the same studies. A signal-to-noise ratio equal to 3 was used.

2.5.2. Intra-day accuracy and precision

For the intra-day accuracy and precision experiments, 10 independent spiked serum samples were analyzed both at a concentration of LOQ and at the midpoint of the calibration range. Working solutions A_2 (e.g. tramadol 5000 ng/ml) and B_2 (e.g. meprobamate 100,000 ng/ml) were prepared from standard solution of a certain drug (10.0, 1.0, 0.1 mg/ml) in

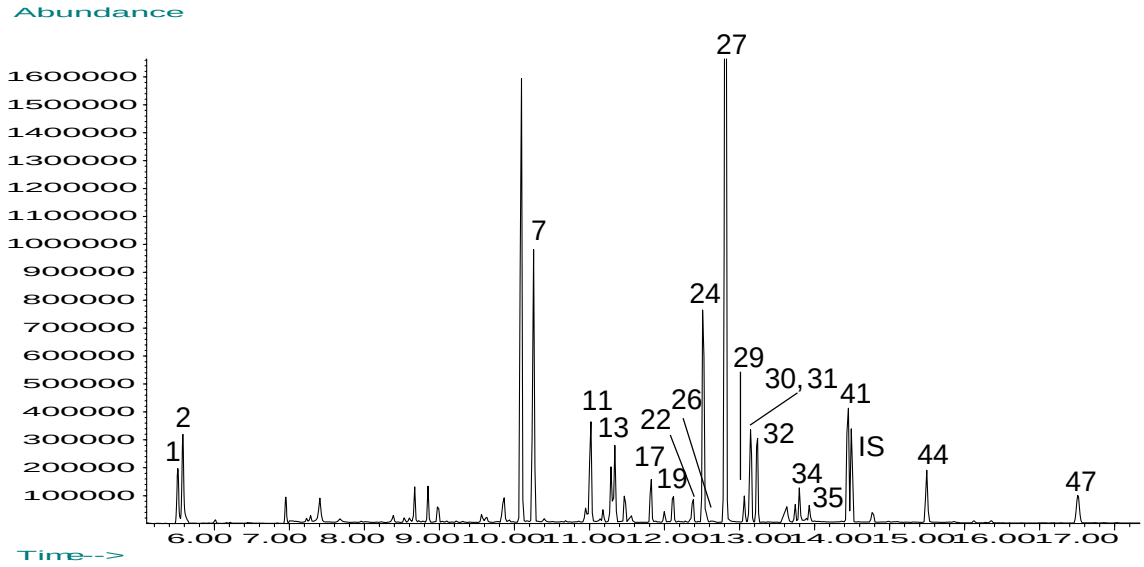

Fig. 2. GC–MS selected ion chromatogram of the spiked serum standard solution B. The concentration levels of the derivatized compounds are the same as the highest standard. For the peak identification, the numbers refer to Table 3. For the instrumental information and temperature program, see Section 2.4.

Table 2
Linearity, limit of detection (LOD) and limit of quantitation (LOQ)

Compound	Tested range (ng/ml)	Linearity range (ng/ml)	r^2[a]	Calibration range (ng/ml)	LOD (ng/ml)	LOQ[b]
GC–MS:						
Meprobamate-?TMS[c]	50–25000	50–25000	0.9916	2000–20000	200	2000
Carisoprodol-?TMS[c]	50–25000	50–12500	0.9953	1000–10000	100	1000
Tramadol-TMS	10–5000	10–500	0.9965	50–500	<10	50
Orphenadrine	10–5000	10–500	0.9972	25–250	10	25
Venlafaxine-TMS	10–5000	10–2500	0.9935	25–250	<10	25
Fluvoxamine-TBDMS	10–5000	50–1000	0.9954	50–500	25	50
10-monohydroxycarb.-?TMS[c]	20–10000	20–2000	0.9995	200–2000	20	200
Methadone	10–5000	10–500	0.9990	25–250	<10	25
Dextropropoxyphene	10–5000	10–500	0.9990	25–250	10	25
Amitriptyline	10–5000	50–1000	0.9961	50–500	25	50
Trimipramine	10–5000	10–2500	0.9979	50–500	25	50
Imipramine	10–5000	10–2500	0.9990	25–250	<10	25
Doxepin	10–5000	50–2500	0.9988	50–500	25	50
Cocaine	1–500	2–500	0.9958	10–100	2	10
Mianserin	5–5000	5–5000	0.9994	25–250	<5	25
Tizanidine-2TMS	10–5000	10–1000	0.9977	25–250	10	25
Mirtazapine	2–1000	2–500	0.9998	25–250	5	25
Fluoxetine-TBDMS	10–5000	25–500	0.9997	25–250	25	50
Promazine	10–5000	10–1000	0.9978	25–250	<10	25
THC-TBDMS	2–1000	10–500	0.9975	10–100	5	10
Clomipramine	10–5000	10–2500	0.9980	50–500	<10	50
Citalopram	10–5000	10–1000	0.9947	25–250	<10	25
Codeine-TMS	2–1000	5–1000	0.9999	10–100	5	10
Nordazepam-TBDMS	10–5000	10–1000	0.9993	100–1000	<10	50
Ethyl Morphine-TMS	2–1000	5–500	0.9986	10–100	2	10
Sertraline-TMS	2–1000	2–500	0.9988	20–200	4	20
Carbamazepine-TBDMS	20–10000	20–500	0.9928	500–5000	50	500
Chlorprotixene	10–5000	10–2500	0.9992	50–500	<10	50
Chlorpromazine	10–5000	10–2500	0.9993	25–250	<10	25
Levomepromazine	10–5000	10–5000	0.9896	50–500	25	50
Diazepam	10–5000	10–1000	0.9912	100–1000	<10	50
Oxazepam-2TBDMS	10–5000	10–1000	0.9963	100–1000	<10	50
Morphine-TMS, TBDMS	2–1000	2–1000	0.9999	5–50	<2	5
Chlordiazepoxide	10–5000	10–2500	0.9897	200–2000	40	200
Phenazepam-TBDMS	n.v.	n.v.	n.v.	10–100	2	10
Midazolam	2–1000	2–1000	0.9999	20–200	2	20
OH-THC-2TBDMS	2–1000	2–200	0.9967	2–20	<2	2
6-Monoacetyl morphine-TBDMS	2–1000	2–200	0.9962	5–50	<2	5
Tematzepam-TBDMS	10–5000	10–2500	0.9982	100–1000	<10	50
Nortriptyline-TBDMS	10–5000	25–2500	0.9931	50–500	25	50
THCC-2TBDMS	2–1000	2–1000	0.9996	2–20	<2	2
Zolpidem	10–5000	10–1000	0.9981	25–250	<10	25
Alprazolam	2–1000	10–1000	0.9952	20–200	10	20
Pholcodine-TMS	2–1000	5–1000	0.9995	10–100	2	10
Thioridazine	10–5000	10–2500	0.9992	50–500	10	50
GC(ECD)						
Lorazepam	2–1000	5–250	0.9982	10–100	5	10
Nitrazepam	4–2000	10–200	0.9892	20–200	10	20
Clonatzepam	2–1000	5–100	0.9927	10–100	5	10
Zaleplon	n.v.	n.v.	n.v.	10–100	<5	10
Triazolam	1–500	2.5–125	0.9936	5–50	5	5
Zopiclone	2–1000	10–500	0.9960	10–100	10	10

n.v.: not validated.

[a] r^2: square of correlation coefficient.

[b] For those compounds that do not fulfill the quantitativity criteria, the value informed here is the same as the lowest standard concentration used for calibration in semi-quantitative screening. For quantitativity of the compounds in serum and whole blood matrices, see Table 5 (serum) and 6 (whole blood). Accuracy as well intra- and inter-day precision within ±20% and 20% R.S.D., respectively, were used.

[c] The number of active hydrogen atoms replaced by the TMS group is not known. 10-Monohydroxycarb.: 10-monohydroxycarbamazepine.

bovine serum. The concentration of each substance in working solution A_2 or B_2 was ten times higher than the highest concentration of calibration standards. Four different concentrations were prepared for calibration of each compound using working standard solutions. For example, the calibration standard concentrations of tramadol were 500, 250, 100, 50 ng/ml (calibration standards A) and meprobamate 20,000, 10,000, 4000, 2000 ng/ml (calibration standards B). The underlined concentrations were used for accuracy and interday precision tests ($n = 10$ each) and all concentrations for calibration. Calibration ranges for all compounds are informed in Table 2, which one can calculate relatively comparable concentrations for each substance. A coefficient of variation (CV) was calculated to evaluate the precision and deviation of the measured concentration compared to the nominal amount to determine accuracy. Accuracy and precision values should not exceed ±20% and 20% relative standard deviation (R.S.D.), respectively, if the compounds can be quantitatively analyzed.

2.5.3. Inter-day precision, extraction efficiency and limit of quantitation

Standard stock solutions A and B, including the same substances as the working standard solutions, were prepared for the interday precision and extraction efficiency experiments as well as for routine analyses. Stock solutions were prepared by weighing 2–40 mg of free compound or their salt to volumetric flasks, except the cannabinoids were pipetted as they were purchased in ampoules. Stock solutions were divided in 0.4 ml aliquots and deep-frozen at −70 °C in acetonitrilic–aqueous media (98:2). Small volume of water was added to increase the solubility of the compounds. In stock solutions A and B the concentration of each compound was ten times higher than the highest calibration standard (see Table 2 for calibration range). Stock solutions were melted and diluted in bovine serum prior to analysis. Quality control (QC) serum samples were also prepared from the same stock solutions in a volumetric flask at low (LOQ) and medium (the midpoint of the calibration range) concentration level for each compound. The aliquots of 1.3 ml were deep-frozen at −70 °C.

Inter-day precision experiments were performed in the same manner as the intra-day studies, except that deep-frozen standard stock solutions and QC samples were used. Validation results were based on daily calibration curves that were updated for each compound in every analysis. Inter-day precision values should not exceed 20% R.S.D. to meet the quantitativity criteria.

In order to define extraction efficiency, three blank serum samples and 10 spiked serum samples at the medium concentration level for each compound were normally extracted. After extraction and separation of the organic layer (4.5 ml), an appropriate quantity of the acetonitrilic stock solution was added to the blanks. Consequently, 9/10 of the standard solution added to the spiked serum samples was added to the blanks after the extraction procedure. The mean results were compared to each other, and the recovery percentage was calculated.

The limit of quantitation (LOQ) was set on the basis of the relevant concentrations of each substance and the accuracy as well intra- and inter-day precision within ±20% and 20% R.S.D., respectively. In addition, matrix effects were studied for each compound in matrix testing experiment (see Section 2.5.5) and should not exceed the above mentioned criteria. The compounds exceeding criteria are semiquantitatively, but not quantitatively, analyzed.

2.5.4. Selectivity

Ten different blank whole blood samples were systematically analyzed to check possible selectivity problems and baseline interference with GC–MS and GC (ECD). All target ion traces of SIM are presented and compared to blank whole blood sample. Blank serum sample was analyzed in every validation test and in routine analysis.

2.5.5. Matrix testing

For estimation of matrix effects, five different authentic whole blood samples, that were confirmed negative, were spiked and analyzed using serum calibration standards that were prepared from deepfrozen stock solutions. The concentration of each compound was equal to the midpoint of the calibration range. Both accuracy and precision values were calculated and taken into consideration, when evaluating quantitativity of the method for each compound. Accuracy and precision values should not exceed ±20% and 20% R.S.D., if the compounds are quantitatively determined in whole blood.

3. Results and discussion

3.1. Derivatization

As an essential part of a successful drug analysis, the optimization of the derivatization reaction was emphasized. In our preliminary tests, and our previous experience, silylation had the best properties for large-scale screening with a variety of substances, due to its superior derivatization power as well as stable and intensive derivatives, apart from the structural differences of the analyzed compounds.

The TBDMS ethers formed by MTBSTFA possess superior properties compared to other silylated derivatives, e.g. their hydrolytic stability is greater and thus sensitivity towards moisture, in addition to more specific mass fragmentation and higher *m*/*z* values in EI-MS spectra [21]. These properties have been studied thoroughly and have been largely proven in practice [22–24]. On the other hand, the derivatization of sterically hindered groups has been found to be more problematic due to the larger TBDMS group to be attached than in trimethylsilylation and TMS derivative formation [21]. We maximized the benefits of both reagents while minimizing the disadvantages. To our

knowledge, there are no reports demonstrating systematically validated and optimized combination of MTBSTFA and MSTFA. The results of using two reagents were compared to using MTBSTFA alone, the other reaction parameters being equal.

3.1.1. Derivatization solvent

For a few years, silylation reactions have been carried out successfully in acetonitrilic media in our laboratory. These experiments are consistent with the literature and systematic research work [24].

3.1.2. The addition order of derivatization reagents, heating time and temperature

Minimum byproducts and partial derivatization occurred by adding MTBSTFA prior to MSTFA. The other alternatives increasingly resulted in incomplete and uncontrollable reactions. Strong heating was needed to successfully complete the first step of the derivatization reaction with MTBSTFA. Heating times of 15, 30 and 60 min and temperatures of 55 and 80 °C were tested with triplicate standard serum samples including tramadol, codeine, THC, oxazepam, morphine and temazepam, as these represent a few derivatized, core substances of the analyzed compounds. MSTFA was used normally without heating. A lower temperature (55 °C) was not enough to completely derivatize the sterically hindered groups, such as the hydroxyl group in temazepam, although it seemed to be enough to totally derivatize the hydroxyl group of THC, the 6-OH group of morphine, and both the secondary amine and hydroxyl group of oxazepam already in 15 min. There were no changes in the responses of tramadol and codeine due to their nature of derivatizing by MSTFA to the TMS derivatives. No increased responses were observed between 30 and 60 min heating at 80 °C, so 30 min seemed to be enough to carry out the reactions as completely as possible. Therefore, a temperature of 80 °C for 30 min was chosen to guarantee the best possible MTBSTFA derivatization.

3.1.3. Volume of reagents

In the preliminary tests, 15 μl of both derivatizing reagents were sufficient. To guarantee the excess of reagents, 15, 30 and 50 μl of both reagents were added to the serum standard including the substances of standard stock solution A. In addition, three negative whole blood samples spiked with the same amount of compounds were used to take into account the possible matrix effects. The concentrations were equal to the highest standard of solution A. All samples were tested in triplicate to minimize random errors. In conclusion, 15 μl of both reagents were noted to be sufficient.

3.1.4. Using two reagents versus MTBSTFA used alone

At least two important aspects should be noted in two-reagent use. First, the first derivatization reaction should be as complete as possible prior to introducing the second reagent to avoid unwanted partial derivatization; strong heating was thus mandatory with MTBSTFA. Second, the samples must be allowed to cool down to room temperature (10–15 min) before adding the second reagent (MSTFA). This strongly affects the accuracy, precision and linearity of several compounds, especially tizanidine, carisoprodol, meprobamate, sertraline, fluvoxamine and tramadol. But the improvements can be clearly seen throughout the derivatized substances.

A separate SIM program was carried through to evaluate the performance of the method by using only MTBSTFA as a derivatizing reagent. Several advantages were nevertheless obtained by using two reagents. First, the OH group of tramadol and venlafaxine can not be derivatized (at least in these conditions) to TBDMS derivatives. An interfering peak broadening was observed by analyzing the underivatized forms, especially with venlafaxine. The structural similarities of these compounds should be noted, although they have different clinical uses. Second, the 6-OH group of morphine was only partially derivatized with MTBSTFA. Apparently, the larger TBMDS group does not have sufficient space for the replacement reaction of the active free hydrogen atom, likely due to the steric hindrance of the bridge structure relative to the 6-OH group in the morphine molecule. The sensitivity of morphine was consequently drastically decreased. A stable and unique morphine derivative was achieved by the two-reagent use. 6-OH derivatized with MSTFA, a smaller TMS group clearly not suffering from steric hindrance, and 3-OH with MTBSTFA. Third, the total runtime of GC–MS separation was approximately 2 min shorter with two reagents due to the long elution time of pholcodine-TBDMS. In two-reagent use, pholcodine appeared completely in TMS form. Fourth, the retention times of the analyzed compounds were more equally divided among the entire runtime of GC–SIM–MS separation. Constructing the SIM program was easier and allowed more space in the SIM windows to add possible new substances. Finally, looking at the matter from different viewpoint, valuable information was obtained on the behaviour of various drug classes in silylation reactions and the stable derivative formation.

Two disadvantages were noted: the enol form of methadone was derivatized in a few days to TMS derivative in the sample vials, and thus only semiquantitative results were obtained. 11-OH-THC was only partially derivatized to 2TBDMS derivative. Another 11-OH-THC derivative was TMS, TBDMS form. It seems that even more drastic conditions are needed with MTBSTFA to completely derivatize also the other OH group of 11-OH-THC.

3.2. Selected ion monitoring (SIM)

Due to the high number of the analyzed substances, it is obvious that a variety of SIM ion windows had to be created at corresponding time intervals. Accordingly, the substances were divided into six different time windows. The number of time windows was a compromise between sensitivity and practicability. More windows allow one to include fewer

ions in each window, enabling greater sensitivity, but this complicates the optimization of SIM in day-to-day analyses.

The most intensive SIM ions have been chosen for each compound based on scanning mode experiments, excluding only few compounds with possible background interference that was carefully checked. Generally, ions with a small molecular mass (<100 *m*/*z*) have been avoided to a certain extent, due to usual increment of background interference and risen baseline, especially in whole blood. Three characteristic ions were selected for each compound. For dextropropoxyphene (*m*/*z* 58) and flurazepam (*m*/*z* 86, IS) only one ion was chosen because of the insignificant responses of other ions. However, for most of the compounds, one quantitation ion and two qualifier ions were selected without difficulty. Furthermore, the valuable sensitivity increments in higher *m*/*z* values were obtained by using target tuning instead of autotuning macro.

Dwell times of 30, 50, 75, 100, 150, 200 and 300 ms were tested, and optimized values were used for each SIM window. The dwell times ranged from 30–75 ms per ion. The changes in dwell times in windows 1 and 6 with only a few ions (4 and 8) did not greatly greatly influence the sensitivies or peak shapes. A more dramatic change was perceived in windows 2–5 having more ions (22–26). The sensitivities of the compounds were drastically decreased, and the peak shapes had become worse in the values higher than 50 ms. The created GC–SIM–MS program and optimized dwell times are given in Table 3.

A Detailed Quant Report was automatically obtained for each sample, and the compounds were identified according to retention times, target ions and relative abundance of qualifier ions compared to the target ion. In addition, all data were manually checked for background interference and false peak selection. An example of the Detailed Quant Report is shown in Fig. 3.

Semiquantitative/quantitative results were obtained by plotting the peak height ratios of every single compound to the internal standard (flurazepam), and comparing them to the standard straight lines of four-point standard serum calibration. The lowest concentration level of a calibration standard to each compound was the same as the accepted cut-off value. Flurazepam was suitable for internal standard, because it is not therapeutically used in Finland. In addition, flurazepam is usually present in whole blood only to a minor extent, even if used. For example, after 15 and 30 mg flurazepam oral dose the mean plasma concentrations reached average peak levels of <2.0 and 2.1 ng/ml at 30 min and 1 h, respectively, [25,26]. These concentrations are insignificant in terms of method performance if one takes into account the concentration of IS, i.e. 1000 ng/ml per sample. However, in extreme intoxication cases the significant interferences are possible, even though unlikely, but should be taken into consideration in countries, where flurazepam is abused. At that case, for example, deuterated benzodiazepines would be a safer choice for internal standard.

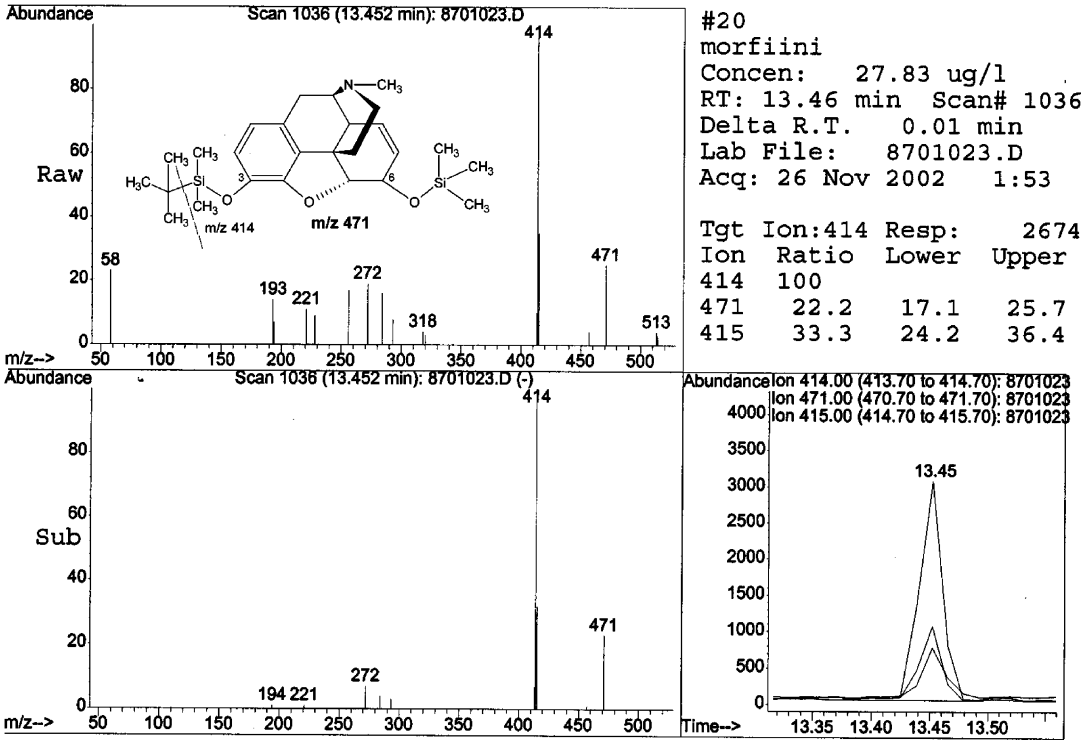

Fig. 3. An example of morphine-positive (27.8 ng/ml) whole blood extract of a driver suspected of intoxication. Mass spectra of morphine-TBDMS, TMS illustrated in the routinely used Detailed Quant Report. The molecular structure of morphine-TBDMS, TMS has been added afterwards.

Table 3
Active groups of compounds and SIM parameters

No.	Compound	Active groups[a]	Retention time (min)	Time window (min)	Dwell time (ms)	SIM ions[b] (m/z)
1	Meprobamate-?TMS[c]	2x $–NH_2$	5.51	4.00–7.00	75	206, 190 (106.4)
2	Carisoprodol-?TMS[c]	2x –NH–, $–NH_2$, –COOH	5.58			160, 176 (80.8)
3	Tramadol-TMS	–OH	8.67	7.00–11.50	30	58, 245 (11.5)
4	Orphenadrine	–	9.09			58, 165 (19.6), 178 (10.1)
5	Venlafaxine-TMS	–OH	9.53			58,134 (5.8)
6	Fluvoxamine-TBDMS	$–NH_2$	10.06			258, 200 (73.1), 144 (93.7)
7	10-Monohydroxycarb.-?TMS[c]	$–NH_2$, –OH	10.26			193, 283 (31.3), 282 (28.7)
8	Methadone[d]	(–C=O)	10.44			72, 165 (9.1), 178 (9.5)
9	Dextropropoxyphene	–	10.62			58
10	Amitriptyline	–	10.96			202, 203 (75.9)
11	Trimipramine	–	11.02			249, 193 (165.7), 208 (54.4)
12	Imipramine	–	11.20			234, 193 (209.6)
13	Doxepin	–	11.29			58, 202 (10.7), 219 (8.8)
14	Cocaine	–	11.38			182, 303 (29.8), 272 (11.5)
15	Mianserin	–	11.43			193, 264 (14.7), 220 (6.5)
16	Tizanidine-2TMS	2x –NH–	11.72	11.50–12.65	30	240, 214 (103.2), 362 (59.5)
17	Mirtazapine	–	11.83			195, 194 (34.7), 208 (19.9)
18	Fluoxetine-TBDMS	$–NH_2$	11.85			219, 268 (119.6), 202 (42.9)
19	Promazine	–	12.13			284, 238 (80.7)
20	THC-TBDMS	–OH	12.24			371, 428 (41.2)
21	Clomipramine	–	12.33			268, 269 (106.9), 58 (96.7)
22	Citalopram	–	12.38			58, 238 (20.9), 208 (18.6)
23	Codeine-TMS	–OH	12.48			371, 234 (54.7)
24	Nordazepam-TBDMS	–NH–	12.52			327, 329 (38.2)
25	Ethyl Morphine-TMS	–OH	12.56			385, 234 (84.3)
26	Sertraline-TMS	–NH–	12.57			274, 276 (85.7)
27	Carbamazepine-TBDMS	$–NH_2$	12.82	12.65–13.50	30	193, 293 (16.7), 194 (16.2)
28	Chlorprotixene	–	13.03			58, 221 (54.0)
29	Chlorpromazine	–	13.07			318, 272 (75.2), 320 (38.9)
30	Levomepromazine	–	13.14			58, 228 (44.8)
31	Diazepam	–	13.16			256, 284 (70.0), 221 (32.5)
32	Oxazepam-2TBDMS	–NH–, –OH	13.24			457, 513 (34.6), 514 (28.9)
33	Morphine-TMS, TBDMS	2x –OH	13.44			414, 471 (28.1), 415 (35.0)
34	Chlordiazepoxide[e]	–NH–	13.81	13.50–16.00	30	283, 282 (120.1), 284 (56.1)
35	Phenazepam-TBDMS	–NH–	13.94			407, 405 (69.6)
36	Midazolam	–	14.00			310, 312 (34.9), 325 (23.7)
37	Lorazepam-2TBDMS	–NH–, –OH	14.02			491, 515 (47.1)
38	Nitrazepam-TBDMS	–NH–	14.12			338, 292 (23.6)
39	OH-THC-2TBDMS	2x –OH	14.14			413, 414 (34.5)
40	6-MAM-TBDMS	–OH	14.38			342, 441 (40.6), 384 (38.2)
41	Tematzepam-TBDMS	–OH	14.45			357, 283 (83.0), 359 (39.1)
42	Nortriptyline-TBDMS	–NH–	14.58			364, 202 (105.6)
43	THCC-2TBDMS	–COOH, –OH	14.99			413, 515 (131.3), 414 (34.5)
44	Zolpidem	–	15.50			235, 307 (9.0), 236 (19.0)
45	Alprazolam	–	16.88	16.00-	50	279, 204 (73.1), 308 (43.5)
46	Pholcodine-TMS	–OH	17.06			100, 114 (117.4)
47	Thioridazine	–	17.51			98, 370 (58.4), 85 (30.6)
	Fluratzepam, IS	–	14.50	13.50–16.00	30	86

[a] Active groups include free hydrogen atoms in the molecular structure of the compound. These hydrogen atoms can be—at least in theory—derivatized.
[b] Values in parentheses are the relative abundances of qualifier ions in respect to the quantitation ion.
[c] The number of active hydrogen atoms replaced by the TMS group is not known. 10-monohydroxycarb.: 10-monohydroxycarbamazepine.
[d] Methadone has the carbonyl group that exists also in enol form. The enol form is vulnerable to derivatization as noted in Section 3.1.4.
[e] Chlordiazepoxide has a free hydrogen atom in the secondary amine group, but does not derivatize in the developed method, likely due to steric hindrance. Consequently, the analyzed form is underivatized.

3.3. GC (ECD) separation

It should be noted that the standard solution A contains all the compounds that are analyzed with the GC (ECD) system. Therefore, standard solution B need not be analyzed with GC. All the benzodiazepines are also seen in ECD, due to their chlorinated, fluorinated and nitro groups containing chemical structures of high electron affinity. The retention times of the analyzed drugs are given in Table 4. The use of gas chromatography–negative-ion chemical ionization–mass

Table 4
Retention times (t_R) of the underivatized compounds in GC(ECD) separation

Compound	t_R (min)
Oxazepam[a]	4.017
Lorazepam[b]	4.463
Diazepam[a]	4.593
Nordazepam[a]	5.240
Midazolam	5.431
Temazepam 1[a,c]	5.620
Flurazepam, IS	5.941
Temazepam 2[a]	6.148
Phenazepam[a]	6.405
Nitrazepam[b]	6.917
Chlordiazepoxide[a]	7.322
Clonazepam[b]	7.388
Temazepam 3[a]	7.604
Alprazolam	7.852
Zaleplon[b]	8.209
Triazolam[b]	8.605
Zopiclone[b]	9.210

[a] The compounds in standard solution B. The peaks of these substances are easily recognized when analyzing blood samples in ECD, if positive. As a result, double-screening of the benzodiazepines is obtained.
[b] These substances are quantitated from GC (ECD).
[c] The most intensive peak of temazepam.

spectrometry (GC–NICI–MS) can be used instead of GC (ECD) for benzodiazepines to attain high sensitivity and improved selectivity, if available.

3.4. Validation procedure

3.4.1. Accuracy, intra- and interday precision

The data on accuracy, intra- and interday precision, are presented in Table 5. Accuracy values varying from chlorprotixene (0.4%) to sertraline (39.3%) at the midpoint of the calibration range, and from ethyl morphine (0.04%) to chlordiazepoxide (44.7%) at a concentration level equal to LOQ, excluding tizanidine at high values (66.4 and 130.7%). The corresponding intra-day precisions were from 6-monoacetylmorphine (2.5%) to fluvoxamine (21.8%), and from midazolam (2.3%) to fluvoxamine (22.2%). The inter-day precisions were all below 31.2% (tizanidine) at both tested levels.

A few compounds that have free hydrogen atoms in active groups, and can thus be derivatized, have high accuracy and precision values. This clearly indicates unstable conditions in the derivazation reaction. A simple solution was observed after the validation tests. The cooling process prior to addition of the second silylating reagent (MSTFA) significantly improved the accuracy, precision and linearity as well as stabilized the derivazation procedure as noted in Section 3.1.4. Improvements in the accuracy and precision of these compounds could be achieved. In addition, it should be noted that for example structurally morphine-like opiates, i.e. morphine, 6-MAM, codeine and pholcodine, have a good accuracy and precision, even when MSTFA is added immediately after the heating process. This indicates that in morphine-like structures complete, stable and reproducible derivatization reaction is achieved rapidly in both conditions, as well as strong binding of TBDMS to the 3-OH group and TMS to the 6-OH group, if available.

3.4.2. Extraction

The calculated mean percentages of extraction recovery are given in Table 5. Especially when one takes into account the quantity of compounds, which range from lipophilic to moderately polar and differ in their ionic properties, the recovery levels were high, except THC. The recovery of THC was surprisingly low, indicating its strong binding to serum proteins. In LLE, this is exceptional, because the protein-bound fraction is usually also extracted. Furthermore, it was noted that the extraction efficiency is better from human whole blood, indicating that the method can be used only for semiquantitative screening purposes of THC in its present form, as the calibration standards are done in bovine serum (the same fact can be observed from matrix testing experiment, see Section 3.4.5). Dissimilar binding behaviour of THC during the extraction in serum and whole blood is likely due to the protein concentration difference between the matrices. The low recovery explains why the LOD value of THC is relatively high, as compared to 11-OH-THC and THCC. The extraction step itself was found to be very rapid, easy to operate, and the emulsion formation was completely avoided.

3.4.3. Linearity, limit of detection (LOD) and limit of quantitation (LOQ)

The method showed good linearity despite the large number of substances with different chemical structures and concentration levels. The linearity ranges in the GC–MS separation were much greater than in the GC (ECD) part. An overview of LOD and LOQ values clearly shows the sensitivity of the method for analyzing low concentrations throughout (see Table 2). In addition, the calibration ranges of each substance used in the validation tests and routine analysis are listed in Table 2.

3.4.4. Selectivity and standard solutions

One selectivity problem was observed in the ECD part of the analysis. As chlordiazepoxide and clonazepam have nearly the same retention time, clonazepam can not be identified reliably if the sample also contains chlordiazepoxide, which can be determined by the GC–MS part. With this method, clonazepam-TBDMS can not be determined by GC–EI–MS either, owing to the interfering matrix component of whole blood. The only selectivity problem in GC–MS (in addition to clonazepam) was noted with levomepromazine. A small matrix component of whole blood that eluates with the same retention time, does not interfere with the screening, but the quantitative results can be slightly higher especially near the cut-off level. Levomepromazine was validated using a cut-off value of 25 ng/ml, but the cut-off was raised to 50 ng/ml to avoid any problems.

Table 5
Accuracy, intra- and inter-day precision of the method, as well as recovery of extraction ($n = 10$)

Compound[a]	Nominal concentration (ng/ml)	Accuracy (%)	Intra-day precision (%)	Inter-day precision (%)	Recovery (%)
GC–MS					
Meprobamate-?TMS[b]	2000/10000	6.5/24.9	10.8/13.4	16.0/22.5	99.1
Carisoprodol-?TMS[b]	1000/5000	9.5/8.9	10.7/11.4	12.1/22.1	92.9
Tramadol-TMS	50/250	26.1/1.2	9.2/10.6	19.4/15.1	95.7
Orphenadrine	25/125	20.7/3.0	9.6/8.9	18.8/15.5	101.7
Venlafaxine-TMS	25/125	40.6/18.4	9.8/5.7	29.8/21.7	115.5
Fluvoxamine-TBDMS	50/250	9.6/13.2	22.2/21.8	19.3/15.2	104.2
10-monohydroxycarb.-?TMS[b]	200/1000	17.6/7.0	5.7/6.8	11.8/17.0	88.1
Methadone	25/125	24.9/15.5	6.8/11.0	16.5/10.4	81.6
Dextropropoxyphene	25/125	26.8/4.9	12.3/8.3	14.3/12.1	90.7
Amitriptyline*	50/250	19.8/1.3	5.7/5.1	14.1/8.5	92.6
Trimipramine*	50/250	6.7/1.5	7.1/10.6	14.2/8.4	78.7
Imipramine	25/125	22.1/2.0	9.3/6.4	12.7/11.3	89.8
Doxepin*	50/250	12.2/8.9	7.2/8.7	10.8/7.8	103.3
Cocaine	10/50	n.v.[c]/8.6	n.v.[c]/10.5	21.5/10.8	91.6
Mianserin*	25/125	1.8/10.7	16.9/10.7	11.5/8.2	93.6
Tizanidine-2TMS	25/125	130.7/66.4	13.2/18.2	28.4/31.2	89.0
Mirtazapine*	25/125	0.8/5.3	7.0/6.8	12.9/8.0	98.2
Fluoxetine-TBDMS*	50/250	5.2/1.9	8.3/9.5	13.8/8.2	79.2
Promazine*	25/125	0.2/6.0	7.2/4.4	10.5/14.0	95.5
THC-TBDMS	10/50	10.6/3.7	6.1/6.3	23.3/17.6	15.4
Clomipramine*	50/250	2.4/4.4	7.4/5.8	2.5/7.4	77.8
Citalopram	25/125	26.2/5.5	8.0/8.5	8.6/9.5	103.3
Codeine-TMS*	10/100	6.5/2.9	5.0/5.3	9.2/7.1	100.0
Nordazepam-TBDMS*	100/500	2.2/1.3	3.7/6.4	8.3/9.3	98.9
Ethyl Morphine-TMS*	10/50	0.04/4.4	4.8/6.2	11.9/9.3	103.0
Sertraline-TMS	20/100	27.4/39.3	11.0/11.5	15.8/10.7	93.3
Carbamazepine-TBDMS*	500/2500	19.8/8.0	11.8/9.5	8.4/10.5	99.7
Chlorprotixene	50/250	25.1/0.4	6.2/3.7	11.9/10.9	73.5
Chlorpromazine*	25/125	1.0/5.8	8.4/4.8	9.3/9.0	83.6
Levomepromazine	25/125	12.0/8.7	12.2/7.7	8.2/10.6	79.3
Diazepam*	100/500	16.6/9.0	6.4/8.5	9.8/9.3	97.9
Oxazepam-2TBDMS*	100/500	6.3/2.2	3.7/9.5	9.9/14.0	99.9
Morphine-TMS, TBDMS*	5/25	0.5/0.6	3.3/5.0	13.8/14.7	60.3
Chlordiazepoxide	200/1000	44.7/39.0	19.3/17.0	17.0/20.2	101.3
Phenazepam-TBDMS	n.v.[c]	n.v.[c]	n.v.[c]	n.v.[c]	n.v.[c]
Midazolam*	20/100	14.7/1.7	2.3/3.3	15.5/14.2	93.3
OH-THC-2TBDMS	2/10	6.8/9.6	7.0/10.9	21.6/13.2	78.9
6-Monoacetyl morphine-TBDMS*	5/25	14.4/1.4	5.4/2.5	7.5/6.0	89.9
Tematzepam-TBDMS*	100/500	20.3/5.4	7.7/6.0	7.7/9.8	99.8
Nortriptyline-TBDMS*	50/250	2.7/5.1	7.4/2.8	11.5/18.3	95.8
THCC-2TBDMS*	2/10	5.1/2.9	4.2/4.4	19.3/10.5	72.6
Zolpidem*	25/125	2.3/6.8	6.3/4.8	9.2/10.8	85.8
Alprazolam*	20/100	5.8/3.0	12.8/13.1	13.1/16.6	88.0
Pholcodine-TMS	10/50	22.0/0.9	3.1/4.1	12.3/10.4	73.0
Thioridazine*	50/250	8.6/0.7	7.9/8.6	11.1/11.7	62.9
GC(ECD)					
Lorazepam*	10/50	14.8/2.1	3.5/3.8	4.7/9.3	66.9
Nitrazepam*	20/100	9.4/0.7	3.0/3.3	8.7/10.4	105.1
Clonatzepam*	10/50	6.0/3.8	3.3/2.6	5.1/4.1	69.4
Zaleplon	n.v.[c]	n.v.[c]	n.v.[c]	n.v.[c]	n.v.[c]
Triazolam*	5/25	9.4/9.7	3.9/2.7	5.0/4.6	77.5
Zopiclone*	10/50	11.9/1.9	3.2/3.4	5.9/9.4	78.8

[a] The compounds marked with an asterisk can be simultaneously quantitatively analyzed from serum samples. The accuracy values were not allowed to exceed 20% and the intra-/inter-day precisions are lower than 20% R.S.D. on both validated concentrations.

[b] The number of active hydrogen atoms replaced by the TMS group is not known. 10-monohydroxycarb.: 10-monohydroxycarbamazepine.

[c] n.v.: not validated.

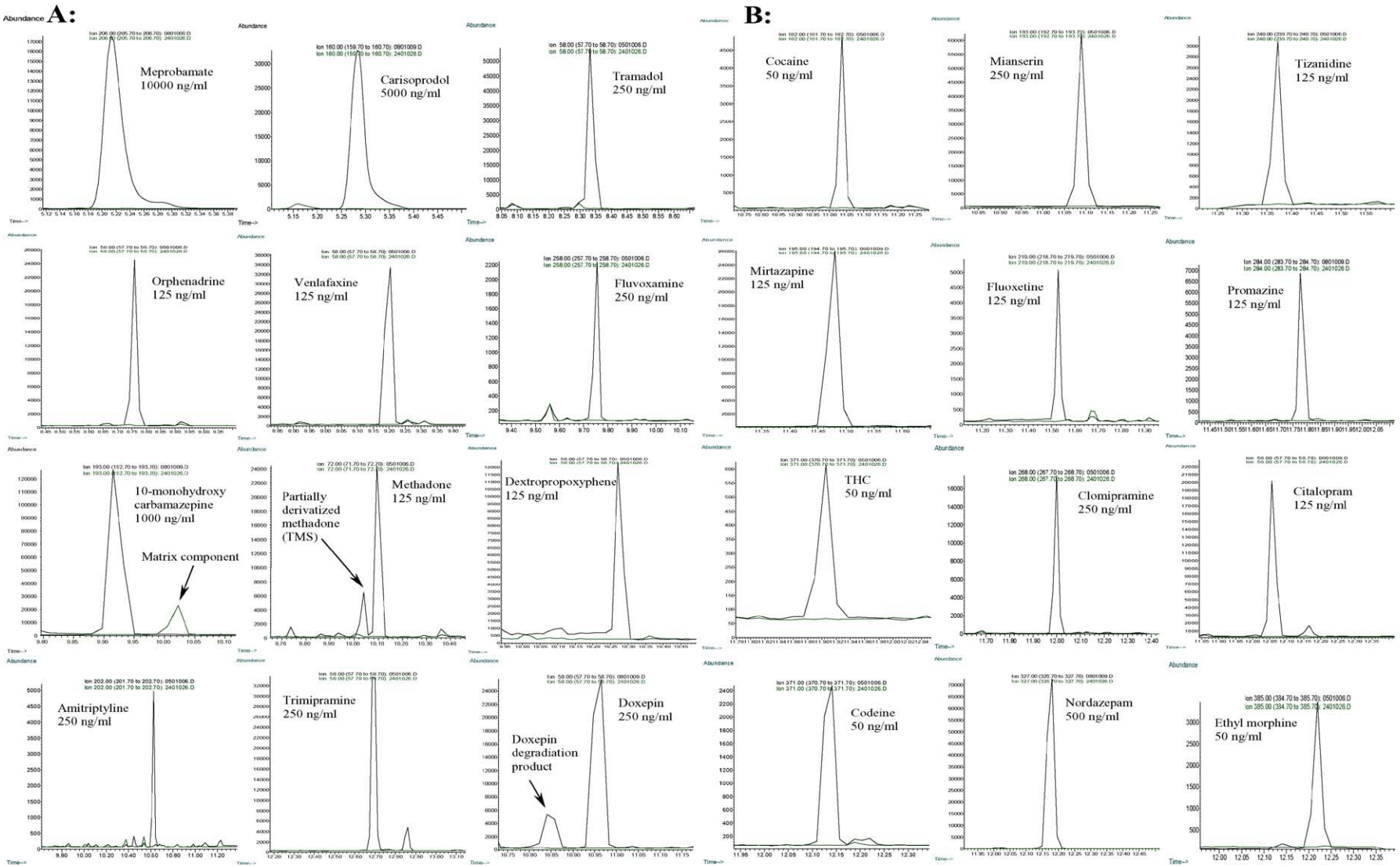

Fig. 4. Selected ion overlay GC–EI–MS chromatograms of the spiked bovine serum sample (concentration is equal to the midpoint of the calibration range i.e. 5× LOQ excluding fluoxetine that is 2.5× LOQ) and blank human whole blood sample. Target ion of each compound is used to show the selectivity of the method. (A) Compounds 1–13 (excluding imipramine). (B) Compounds 14–25. (C) Compounds 26–37. (D) Compounds 38–47. Numbers refer to Table 3.

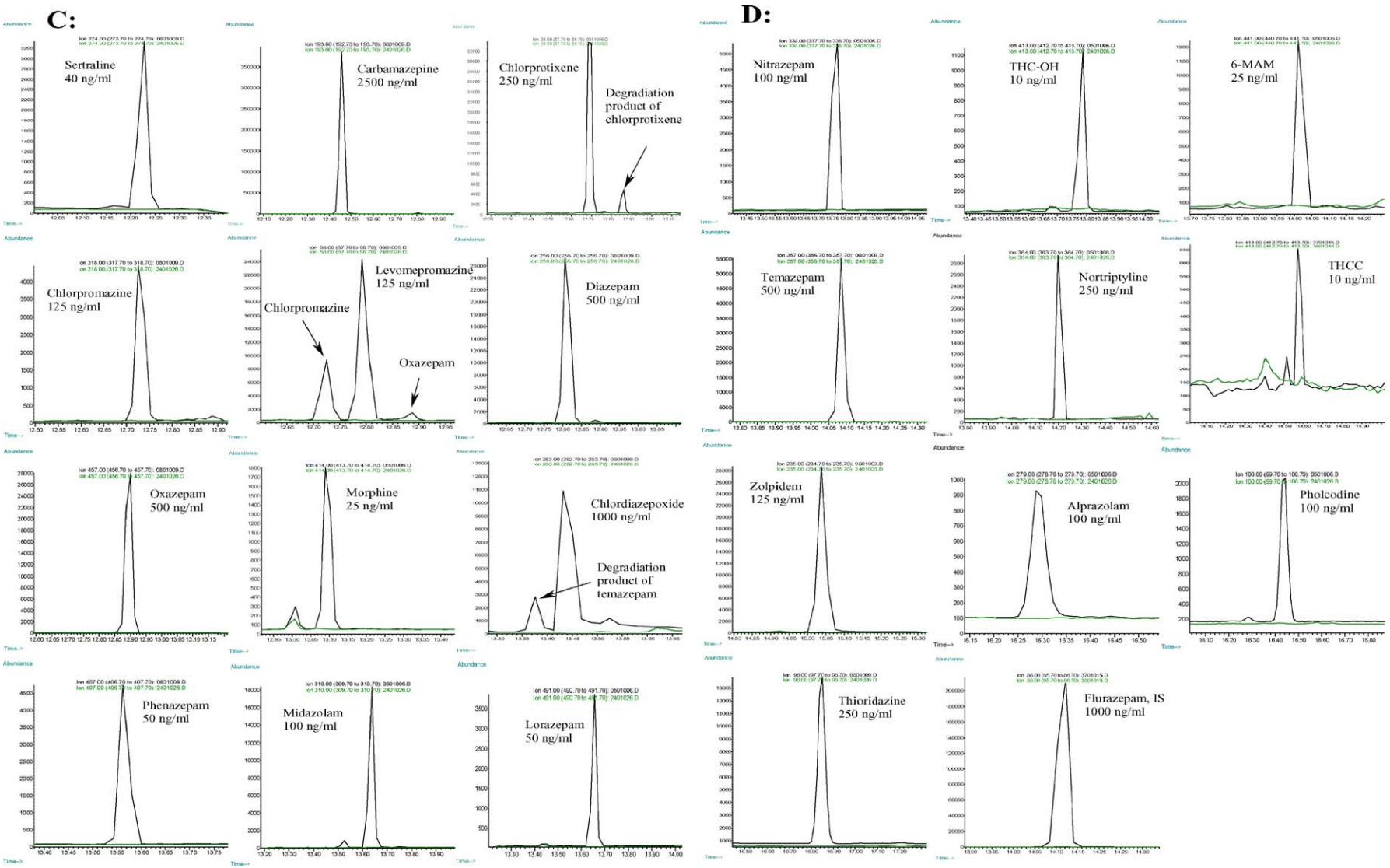

Fig. 4. (*Continued*).

Table 6
Matrix testing

Compound[a,b]	Nominal concentration (ng/ml)	Accuracy/ precision (%)
GC–MS		
Meprobamate-?TMS	10000	−41.1/8.85
Carisoprodol-?TMS*	5000	−2.70/3.08
Tramadol-TMS*	250	−1.53/5.93
Orphenadrine	125	−20.4/6.33
Venlafaxine-TMS*	125	−16.5/5.72
Fluvoxamine-TBDMS*	250	16.0/3.86
10-monohydroxy carb.-?TMS*	1000	10.5/4.00
Methadone*	125	4.02/4.77
Dextropropoxyphene*	125	−5.15/6.77
Amitriptyline	250	−29.6/8.64
Trimipramine	250	−8.80/30.5
Doxepin**	250	−13.0/8.44
Cocaine*	50	−8.70/9.72
Mianserin**	125	−5.77/9.48
Tizanidine-2TMS	125	−37.1/13.1
Mirtazapine**	125	−1.30/4.63
Fluoxetine-TBDMS**	125	18.6/7.94
Promazine**	125	−14.4/3.20
THC-TBDMS	50	69.8/6.09
Clomipramine	250	−22.2/3.63
Citalopram*	125	9.00/4.43
Codeine-TMS**	100	−19.4/4.65
Nordazepam-TBDMS**	500	−3.80/7.15
Ethyl Morphine-TMS	50	−25.2/2.01
Sertraline-TMS	100	−23.8/8.05
Carbamazepine- TBDMS**	2500	−0.80/4.96
Chlorprotixene*	250	−10.3/3.80
Chlorpromazine**	125	−11.0/3.52
Levomepromazine*	250	−5.60/5.00
Diazepam**	500	6.90/4.14
Oxazepam-2TBDMS**	500	−4.60/1.41
Morphine-TMS, TBDMS	25	−37.4/5.83
Chlordiazepoxide	1000	−17.3/21.1
Phenazepam-TBDMS*,c	50	−5.60/6.66
Midazolam**	100	−17.2/6.79
OH-THC-2TBDMS	10	−32.2/5.63
6-Monoacetyl morphine-TBDMS**	25	−6.27/4.48
Tematzepam-TBDMS**	500	13.6/5.02
Nortriptyline-TBDMS	250	29.9/6.43
THCC-2TBDMS**	10	−14.0/7.31
Zolpidem**	125	2.00/6.10
Alprazolam**	100	−4.08/9.35
Pholcodine-TMS	50	−30.0/7.74
Thioridazine**	250	−1.80/4.05
GC(ECD)		
Lorazepam	50	−23.7/3.13
Nitrazepam**	100	−12.2/2.04
Clonatzepam**	50	−18.4/2.24
Zaleplon*,c	100	−7.45/0.87
Triazolam**	25	−18.2/1.84
Zopiclone**	50	−0.46/0.80

Accuracy and inter-day precision of spiked whole blood samples ($n = 5$) using serum calibrators.

[a] The compounds marked with two asterisks can be simultaneously quantitatively analyzed in whole blood. These compounds fulfill all the criteria that was set for quantitative analysis: the accuracy values were not allowed to exceed ±20% and the intra-/inter-day precisions are lower than 20% R.S.D. on both validated serum concentrations (see Table 5). In addition, the values in the matrix testing should be lower than above mentioned values.

[b] The compounds marked with an asterisk fulfill the matrix testing criteria for quantitativity, but not all other criteria (see Table 5).

[c] Both of these compounds fulfill the criteria of matrix testing, but were not completely validated. Therefore, the quantitativity of zaleplon and phenazepam is not confirmed.

The matrix component can be avoided for instance in separate quantitative analyses by replacing butyl acetate with toluene as an extraction solvent. Overall, the selectivity of the method was proven sufficient and no other selectivity problems were observed during the validation or routine analyses. All target ion traces of SIM are illustrated in Fig. 4.

Standard stock solutions proved to be stable for at least 6 months when stored in acetonitrile–water (98:2) in the dark at −70 °C. Six month old standard stock solutions A and B were compared to freshly prepared standard stock solutions. All substances were within ±20% of nominal value. In addition, many commercial companies that sell their standard substances in acetonitrile or methanol (1.0, 0.1 mg/ml) guarantee years of stability at 4 °C. 2–40 mg of each pure substance are needed to prepare stock solutions A and B for a 6-month period, i.e. over 100 stock solutions if necessary. 25–30 samples can be analyzed in a day per technician using one of both standard stock solutions A and B.

3.4.5. Matrix testing and quantitativity

Totally 14 compounds did not fulfill the quantitativity criteria that was set (see Table 6). Accuracy values of 12 compounds and precision values of 2 compounds exceeded the limits of ±20% and 20% R.S.D. From these 14 compounds amitriptyline, trimipramine, ethyl morphine, morphine, nortriptyline, pholcodine and lorazepam passed other validation tests. Consequently, the quantitativity of these compounds clearly suffers from matrix effects that are caused using bovine serum as a calibration matrix. Furthermore, it seems that whole blood results are generally little bit smaller than corresponding serum results. Only 10 compounds from 51 exceeded the spiked nominal value in accuracy testing. However, the majority of the compounds were not markedly affected by matrix effects and it seems that significant matrix effects are highly compound specific. Intra-day precision values show that the method is also highly reproducible for whole blood matrix and the results were even better than the corresponding experiments in serum matrix. Especially, low precision values of GC (ECD) analysis should be marked and the fact that only three compounds exceeded even 10% R.S.D. In summary, totally 22 substances can be analyzed quantitatively according to the validation data using serum as a calibration matrix for whole blood samples and 29 substances by analyzing serum samples. The other compounds are reliably identificated and semiquantitative results are obtained. The results of the matrix testing experiment and quantitativity of the compounds in whole blood and serum samples are summarized in Table 6.

4. Conclusions

Despite the differences in chemical structure and a large variety of therapeutic concentration ranges of the analyzed compounds, semiquantitative/quantitative screening was developed for 51 substances. A rapid and reliable method

requiring only a small sample volume allowed highly sensitive analysis, even in the presence of a high matrix content of whole blood. The simultaneous semiquantitative or quantitative identification power equal to separate determination analyses of various drug classes is considered the main advantage of the method. Two silylating reagents, MTBSTFA and MSTFA, were used successfully in the same analysis after detailed optimization. The combination of GC–MS (SIM) and GC–ECD provides further improvement by facilitating the analyses of triazolam, zaleplon and zopiclone, which lack GC–EI–MS sensitivity. The combination also allows double-screening of most of the benzodiazepines with both detection techniques. A comprehensive validation procedure was carried out to demonstrate the practicability of the method, which can be easily expanded to new substances, if necessary. Furthermore, valuable information obtained from the silylated derivatives can be exploited in a separate determination analysis of numerous drugs/drug classes. The method has been routinely used for drivers suspected of intoxication, as well as for clinical samples. Over 1500 samples have been analyzed during 2002–2003 and the method has been accreditated by the Finnish Centre for Metrology and Accreditation (FINAS). It is especially viable for the routinely performed semiquantitative/quantitative screening of clinical toxicology and forensic applications on large numbers of samples.

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