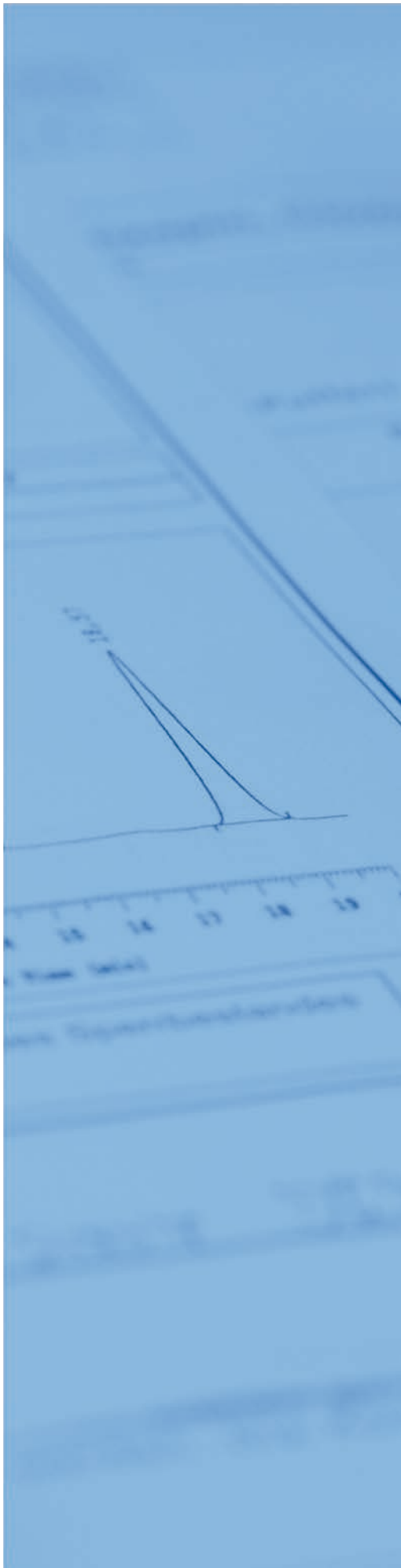
RECIPE

Instruction Manual

ClinMass® TDM Kit System

Benzodiazepines in Serum / Plasma



REF MS9000, MS9500

IVD For in vitro diagnostic use

CE IVDD, 98/79/EC

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1 Introduction

1.1 Information on changes in this instruction manual

This instruction manual (version 2.0) was revised and replaces the previous version 1.0.

Please note the revised reference ranges in section 7.2. The changes are marked on the outer margins.

1.2 Intended use

ClinMass® TDM Kit System

The ClinMass® TDM Kit System is based on a universal TDM Platform (order no. MS9000), which can be used with various Add-On Sets for the Therapeutic Drug Monitoring (TDM) with LC-MS/MS.

The ClinMass® Add-On Set for Benzodiazepines (order no. MS9500) is intended for the determination of Benzodiazepines from human serum or plasma.

The components of the ClinMass® TDM Platform and the ClinMass® Add-On Set for Benzodiazepines have to be used in accordance with the instructions in this user manual. A combination with components from other manufacturers is not intended.

1.2.1 IVD symbols

Symbols according to the EU directive 98/79/EC for in vitro diagnostic medical devices (IVDD), which are used on the product labels and in this user manual:

IVD	For in vitro diagnostic use
	Manufacturer
REF	Order number
LOT	Lot number
	Upper temperature limit: … °C
	Temperature limits: … °C to … °C
	Expiry date: …
	See instructions for use

1.3 Clinical background

Benzodiazepines are a group of psychoactive drugs with a broad range of therapeutic effects. They act as anxiolytics, sedatives and anticonvulsants and belong to the most frequently prescribed drugs all over the world. Currently around 50 different benzodiazepines are globally marketed [1].

Most benzodiazepines share a 5-phenyl-1,3-dihydrobenzo[*e*][1,4]diazepine nucleus with different substituents on the 1, 2, 3, 7 und 2' positions (see Figure 1) [1-3]. Despite this similarity in the chemical structure the drugs differ in pharmacokinetics and metabolic properties.

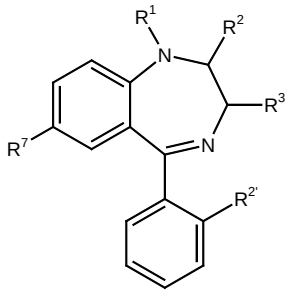


Figure 1. Chemical structure of the 1,4-benzodiazepines [4]

Long-term administration of benzodiazepines may require therapeutic drug monitoring (TDM). The quantification is primarily performed from serum in order to optimise the drug dosing, to verify consumption compliance and to identify changes in pharmacokinetics.

The RECIPE analytical method provides the reliable quantification of 35 different benzodiazepines and metabolites for TDM (see method description in section 1.4). The list of analytes is displayed in Table 1.

Table 1. Analyte list

Analytes		
3-OH-Bromazepam	Demoxepam	Norclobazam
7-Aminoclonazepam	Desalkylflurazepam	Nordiazepam
7-Aminoflunitrazepam	Desmethylflunitrazepam	Oxazepam
7-Aminonitrazepam	Diazepam	Prazepam
α-OH-Alprazolam	Estazolam	Temazepam
α-OH-Midazolam	Flunitrazepam	Tetrazepam
α-OH-Triazolam	Flurazepam	Trazodone
Alprazolam	Lorazepam	Triazolam
Bromazepam	Lormetazepam	Zaleplon
Chlordiazepoxide	Medazepam	Zolpidem
Clobazam	Midazolam	Zopiclone
Clonazepam	Nitrazepam	

1.4 General description of the analytical procedure

The analytical procedure is based on a universal ClinMass® TDM Platform (order no. MS9000), which can be used with various ClinMass® Add-On Sets (ClinMass® TDM Kit System).

The ClinMass® Add-On Set for Benzodiazepines (order no. MS9500) contains the analyte-specific kit components for the determination of 35 different benzodiazepines. Analysis is performed by HPLC with tandem mass spectrometry (LC-MS/MS).

Prior to the LC-MS/MS analysis a short sample preparation is carried out in order to remove the sample matrix and to spike the samples with an internal standard (see sample preparation section 5.2).

The prepared samples are injected into the LC-MS/MS system for chromatographic separation of the compounds. The analytes are then ionised using electrospray ionisation (ESI).

Electrospray ionisation is a soft ionisation technique where a strong electric field is applied to the liquid passing through the ESI-capillary of the MS-source. The ions are mostly preformed in solution before desorption and then transferred into the ion path of the tandem mass spectrometer which consists of three quadrupoles (two mass selectors connected by a collision cell).

Measurement of the analytes is carried out in MRM mode (MRM: Multiple Reaction Monitoring). In this mode only selected ions (known as “precursor ions”) with a defined mass/charge (*m/z*) ratio are isolated in the first quadrupole and subsequently transferred into the collision cell, where they are fragmented by impact with an inert (argon or nitrogen) at defined voltage settings. Among the fragments generated (known as “product ions”) only those with a defined *m/z* ratio can pass the third quadrupole for final detection. In this way the MRM mode ensures a selective identification and quantification of the target analytes.

The analytical method enables a robust and reliable quantitation in complex biological matrices by use of 20 different isotopically labeled internal standards (see section 4.3.2). If required, two transitions are used per analyte (quantifier, qualifier).

Five different ClinMass® Optimisation Mixes are available for the optimisation of the MS/MS parameters (see section 5.3.1) and the test run of the analytical system (see section 5.3.2).

Calibration of the analytical system is performed by use of ClinCal® Serum Calibrators. For this purpose a 4-Level Serum Calibrator Set is available (see section 5.3.3**.**).

Quality control is performed by the use of ClinChek® Serum Controls. These controls are available in two different concentrations (see section 5.3.4).

2 Components of TDM platform and Add-On Set, accessories

2.1 Ordering information

Order No.	Description	Quantity
MS9000	**ClinMass® TDM Platform** for 400 assays	1 pce.
	Content:	
	Autosampler Washing Solution	1 x MS9005
	Mobile Phase A	2 x MS9007
	Mobile Phase B	1 x MS9008
	Sample Preparation Vials	4 x MS9020
	Precipitant P	2 x MS9021
	Separately available components:	
MS9005	Autosampler Washing Solution	1000 ml
MS9007	Mobile Phase A	1000 ml
MS9008	Mobile Phase B	1000 ml
MS9020	Sample Preparation Vials	100 pcs.
MS9021	Precipitant P	25 ml
	Start Accessories:	
MS9030	Analytical Column with test chromatogram	1 pce.
MS9032	Prefilter	1 pce.
	Accessory:	
MS9022	Diluting Solution D	50 ml
MS9500	**ClinMass® Add-on Set** **for Benzodiazepines in Serum / Plasma** for 200 assays	1 pce.
	Content:	
	Internal Standard IS, lyophil.	1 x MS9512
	Serum Calibrator Set, lyophil. (Level 0 - 3)	1 x MS6013
	Manual	
	Separately available components:	
MS9512	Internal Standard IS, lyophil.	5 x 5 ml
MS6013	Serum Calibrator Set, lyophil. (Level 0 - 3)	4 x 1 x 1 ml
	Start Accessories:	
MS6015	Optimisation Mix 1, lyophil.	2 ml
MS6016	Optimisation Mix 2, lyophil.	2 ml
MS6017	Optimisation Mix 3, lyophil.	2 ml
MS6018	Optimisation Mix 4, lyophil.	2 ml
MS6019	Optimisation Mix 5, lyophil.	2 ml
	ClinChek® Controls:	
MS6082	Serum Control, lyophil., Level I, II	2 x 5 x 1 ml

2.1.1 Safety information

Components such as mobile phases and reagents are chemical preparations and may contain hazardous substances. For safety information please consult the respective safety data sheet (SDS) for each component.

The calibrator and control materials are manufactured from human serum. Although the products are tested for the absence of common infection markers, they still should be considered as potentially infectious. For this reason we recommend the product to be handled with the same precautions as patient samples. Detailed safety information is indicated in the respective SDS.

2.1.2 Storage conditions and lifetime of kit components

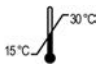
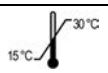
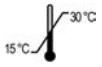
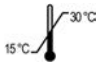
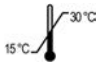
Please unpack all components from the transport packaging **immediately upon receipt** and follow the storage instructions indicated on the product labels and in Table 2.

Unused components, stored under appropriate conditions, can be used until the expiry date indicated on the product label.

After use of ClinMass® Reagents and ClinMass® Mobile Phases the bottles must be closed tightly and stored immediately under the required conditions. Provided that instructions for proper use and storage procedures are followed, the lifetime of the reagents is the same as for the unused products.

Storage conditions and lifetimes of the ClinMass® Internal Standard, the ClinMass® Optimisation Mixes as well as the ClinCal® Calibrators and ClinChek® Controls (lyophilised and after reconstitution) are indicated in the respective product data sheets.

Table 2. Storage conditions of kit components

Order no.		Product description	Storage conditions	
REF	MS9005	Autosampler Washing Solution		Store at 15–30 °C
REF	MS9007	Mobile Phase A		Store at 15–30 °C
REF	MS9008	Mobile Phase B		Store at 15–30 °C
REF	MS9020	Sample Preparation Vials	Store at ambient temperature	
REF	MS9021	Precipitant P		Store at 15–30 °C
REF	MS9022	Diluting Solution D		Store at 15–30 °C
REF	MS9030	Analytical Column		Store at 15–30 °C
REF	MS9032	Prefilter	Store at ambient temperature	

Order no.		Product description	Storage conditions	
REF	MS9512	Internal Standard IS, lyophil.		Store below -18 °C*
REF	MS6013	Serum Calibrator Set, lyophil.		Store at 2–8 °C*
REF	MS6015	Optimisation Mix 1, lyophil.		Store below -18 °C*
REF	MS6016	Optimisation Mix 2, lyophil.		Store below -18 °C*
REF	MS6017	Optimisation Mix 3, lyophil.		Store below -18 °C*
REF	MS6018	Optimisation Mix 4, lyophil.		Store below -18 °C*
REF	MS6019	Optimisation Mix 5, lyophil.		Store below -18 °C*
REF	MS6082	Serum Control lyophil., Level I+II		Store at 2–8 °C*

*Refers to the lyophilised product. For storage conditions after reconstitution please consult the product data sheet.

2.1.3 Disposal of laboratory waste

For disposal laboratory waste should be collected separately according to the different chemical properties. Recommendations for the disposal of the product and the respective packaging are indicated in section 13 of the respective Safety Data Sheet (SDS).

3 Required instruments

The use of this test kit requires an LC system with tandem mass spectrometer (LC-MS/MS) with sufficient sensitivity and evaluation software. Data regarding the suitability of the various LC-MS/MS systems is available upon request (info@recipe.de).

Required LC modules:

- Autosampler
- Binary HPLC pump (Mobile Phases A and B)
- Column heater (40 °C)
- Degasser (optional)

For sample preparation the following laboratory instruments are required:

- Pipettes, pipette tips
- Tabletop centrifuge
- Vortex mixer

4 Operation of the analytical system

4.1 Flushing of the LC system

Connect the LC modules **with exception** of the column. Put the outlet capillary into a safe waste container.

Set the HPLC pump at a flow rate of 1 ml/min and flush the LC system with 10 ml of a 50 : 50 mixture of the Mobile Phases A and B.

Connect the analytical column in the column heater and make sure the flow direction follows the arrow marking on the column!

Please also make sure to use the proper fittings for the connection to the column. A new fitting should be used and adapted to the column. For information regarding the proper connection please contact RECIPE.

4.2 Equilibration of the LC system

After the flushing (see section 4.1) equilibrate as follows:

- Set the HPLC pump at a flow rate of 0.6 ml/min, set the column heater at the temperature of 40 °C and equilibrate the column with 10 ml of a 90 : 10 mixture of the Mobile Phases A and B according to the start conditions of the gradient programme (see Table 4).
- Then **stop the pump** and connect the outlet capillary of the analytical column with the tandem mass spectrometer.

4.3 Starting the analytical system

The following sections provide the parameters for the LC system (see section 4.3.1) and the tandem mass spectrometer (see section 4.3.2). Please consult section 5.3 regarding optimisation, equilibration, test run and calibration of the LC-MS/MS system.

Please consult the user manual of the tandem mass spectrometer to ensure proper handling. User trainings provided by the instrument manufacturer, may also be advisable.

4.3.1 LC parameters

Table 3. LC parameters

HPLC pump (Mobile Phases A, B):	Gradient programme of the binary HPLC pump: See Table 4 Make sure the bottles are closed well to avoid alteration of the retention times, which could occur due to evaporation of the mobile phase components.
Analytical column:	The analytical column and the prefilter are installed in the column heater (40 °C). At a flow rate of 0.6 ml/min the backpressure of the analytical column should not exceed 220 bar. The prefilter should be renewed after 300 injections at the latest. It should also be replaced if the backpressure of prefilter plus column is increased by 10 %.
Autosampler:	Injection volume: 1–10 µl* Injection interval: 6.8 min Needle washing: The injection needle needs to be flushed after sample injection (minimisation of sample carryover). Please refer to the recommended needle wash settings in the instruction manual of the autosampler manufacturer. For flushing please use the autosampler washing solution with order no. MS9005. *depending on the sensitivity of the mass spectrometer

The following gradient programme is used for the binary HPLC pump*:

Table 4. Gradient programme

Time [min]	Mobile Phase A [%]	Mobile Phase B [%]	Flow rate [ml/min]
0.01	90	10	0.6
0.21	90	10	0.6
0.30	76	24	0.6
3.00	74	26	0.6
6.20	40	60	0.6
6.21	15	85	0.6
6.40	15	85	0.6
6.41	90	10	0.6
6.80	90	10	0.6

*Note:
Please note that according to the dead volume of the HPLC system in use an adaptation of the gradient might be necessary.

4.3.2 MS/MS parameters

The mass transitions of the analytes and of the respective isotope-labelled substances in the ClinMass® Internal Standard IS are contained in Table 5.

The assignments of the isotope-labelled substances to the analytes are contained in Table 6.

The indicated mass transitions should be considered as starting points for the optimisation. As the optima may vary slightly between the different MS/MS systems, these have to be determined for the respective system in use (see section 5.3.1).

Table 5. Mass transitions of the analytes and isotope-labelled substances in the IS in ESI positive mode

Analyte / IS	**Quantifier MRM**		**Qualifier MRM**	
	Precursor [amu]	**Product [amu]**	**Precursor [amu]**	**Product [amu]**
3-OH-Bromazepam	332.0	78.1	332.0	287.0
7-Aminoclonazepam	286.1	121.1	286.1	222.1
7-Aminoflunitrazepam	284.1	135.1	284.1	93.0
7-Aminonitrazepam	252.1	121.1	252.1	77.0
α-OH-Alprazolam	325.1	297.1	325.1	205.1
α-OH-Midazolam	342.1	324.0	342.1	168.1
α-OH-Triazolam	359.1	176.1	359.1	331.0
Alprazolam	309.1	205.1	309.1	281.1
Bromazepam	316.0	182.1	316.0	209.1
Chlordiazepoxide	300.1	227.0	300.1	282.1
Clobazam	301.1	259.1	301.1	224.1
Clonazepam	316.1	270.1	316.1	214.1
Demoxepam	287.1	180.0	287.1	105.0
Desalkylflurazepam	289.1	140.0	289.1	226.1
Desmethylflunitrazepam	300.1	198.1	300.1	225.1
Diazepam	285.1	154.1	285.1	193.1
Estazolam	295.1	205.1	295.1	267.0
Flunitrazepam	314.1	268.1	314.1	239.1
Flurazepam	388.2	315.0	388.2	134.0
Lorazepam*	321.0	229.0	321.0	275.0
Lormetazepam	335.0	289.0	335.0	177.0
Medazepam	271.1	91.1	271.1	207.1
Midazolam	326.1	291.1	326.1	223.1
Nitrazepam	282.1	180.1	282.1	236.1
Norclobazam	287.1	245.1	287.1	210.0
Nordiazepam	271.1	140.0	271.1	165.0
Oxazepam**	287.1	269.0	287.1	104.1
Prazepam	325.1	271.1	325.1	140.0
Temazepam	301.1	255.1	301.1	283.0
Tetrazepam	289.1	225.1	289.1	197.1
Trazodone	372.2	120.0	372.2	78.1
Triazolam	343.1	315.0	343.1	239.0
Zaleplon	306.1	236.1	306.1	264.1
Zolpidem	308.2	65.1	308.2	235.1
Zopiclone	389.1	217.0	389.1	245.0

Analyte / IS	**Quantifier MRM**		**Qualifier MRM**	
	Precursor [amu]	**Product [amu]**	**Precursor [amu]**	**Product [amu]**
d_4-7-Aminoclonazepam	290.1	121.1	---	---
d_7-7-Aminoflunitrazepam	291.2	138.1	---	---
d_5-α-OH-Alprazolam	330.1	302.0	---	---
d_4-α-OH-Midazolam	346.1	328.1	---	---
d_4-α-OH-Triazolam	363.1	335.0	---	---
d_5-Alprazolam	314.1	286.1	---	---
d_5-Chlordiazepoxide	305.1	286.1	---	---
d_4-Clonazepam	320.1	274.1	---	---
d_5-Diazepam	290.1	154.1	---	---
d_5-Estazolam	300.1	272.1	---	---
d_7-Flunitrazepam	321.2	275.1	---	---
d_4-Lorazepam	327.0	281.1	---	---
d_4-Midazolam	330.1	295.1	---	---
d_5-Nitrazepam	287.1	185.1	---	---
d_5-Nordiazepam	276.1	140.0	---	---
d_5-Oxazepam	292.1	246.1	---	---
d_5-Prazepam	330.2	276.1	---	---
d_5-Temazepam	306.1	260.1	---	---
d_4-Triazolam	347.1	312.1	---	---
d_6-Zolpidem	314.2	235.1	---	---

Please note:

*see section 7.4.1.

**see section 7.4.2

Table 6. Assignment of the analytes to the isotope-labelled substances in the IS

Analyte	**RT [min]**	**Internal Standard IS**	**RT [min]**
3-OH-Bromazepam	1.50	d_7-7-Aminoflunitrazepam	1.51
7-Aminoclonazepam	1.30	d_4-7-Aminoclonazepam	1.30
7-Aminoflunitrazepam	1.51	d_7-7-Aminoflunitrazepam	1.51
7-Aminonitrazepam	1.24	d_4-7-Aminoclonazepam	1.30
α-OH-Alprazolam	2.75	d_5-α-OH-Alprazolam	2.75
α-OH-Midazolam	3.90	d_4-α-OH-Midazolam	3.90
α-OH-Triazolam	2.74	d_4-α-OH-Triazolam	2.74
Alprazolam	3.67	d_5-Alprazolam	3.67
Bromazepam	2.02	d_5-α-OH-Alprazolam	2.75
Chlordiazepoxide	3.55	d_5-Chlordiazepoxide	3.55
Clobazam	4.48	d_5-Nordiazepam	4.37
Clonazepam	3.44	d_4-Clonazepam	3.44
Demoxepam	2.19	d_7-7-Aminoflunitrazepam	1.51
Desalkylflurazepam	4.12	d_6-Zolpidem	3.50
Desmethylflunitrazepam	2.89	d_5-Oxazepam	3.00
Diazepam	5.14	d_5-Diazepam	5.14
Estazolam	3.11	d_5-Estazolam	3.11
Flunitrazepam	4.13	d_7-Flunitrazepam	4.13
Flurazepam	4.79	d_4-Midazolam	4.99

Analyte	**RT [min]**	**Internal Standard IS**	**RT [min]**
Lorazepam	3.37	d_4-Lorazepam	3.37
Lormetazepam	4.61	d_4-Midazolam	4.99
Medazepam	6.48	d_5-Prazepam	6.23
Midazolam	4.99	d_4-Midazolam	4.99
Nitrazepam	3.06	d_5-Nitrazepam	3.06
Norclobazam	3.23	d_5-Estazolam	3.11
Nordiazepam	4.37	d_5-Nordiazepam	4.37
Oxazepam	3.00	d_5-Oxazepam	3.00
Prazepam	6.23	d_5-Prazepam	6.23
Temazepam	4.24	d_5-Temazepam	4.24
Tetrazepam	5.90	d_5-Prazepam	6.23
Trazodone	4.73	d_4-Midazolam	4.99
Triazolam	3.93	d_4-Triazolam	3.93
Zaleplon	2.52	d_7-7-Aminoflunitrazepam	1.51
Zolpidem	3.50	d_6-Zolpidem	3.50
Zopiclone	2.26	d_5-Chlordiazepoxide	3.55

4.3.2.1 System-specific settings of various MS/MS systems

Device-specific data for the various MS/MS systems by different suppliers is available upon request (info@recipe.de).

4.4 Standby mode

When the analytical system is not in use, the pumps should be switched off, the mobile phases may remain in the LC system.

The vacuum pumps of the tandem mass spectrometer (MS/MS system) should be in permanent operation. In order to protect the ion source and the multiplier the MS/MS system should be switched into standby mode.

For a longer operation pause the analytical column should be disconnected and stored tightly closed. The LC system should then be flushed with a water/acetonitrile mixture (1 + 1).

5 Implementation of the analytical procedure

5.1 Collection and storage of samples

The therapeutic drug monitoring of benzodiazepines is primarily performed from serum. Plasma may be used alternatively.

Serum extraction should not be performed by use of collection tubes with gel separators. Some gels could partially absorb the analytes and thus lead to false low analytical values [5].

At room temperature (15–30 °C) the samples can be stored at least three days. At temperatures between 2–8 °C the samples can be stored at least 7 days and below -18 °C at least three months (multiple freeze-thaw cycles should be avoided).

Zopiclone:
Zopiclone has a shorter sample stability. For the determination the samples have to be frozen immediately after serum or plasma extraction. Stored at temperatures below -18 °C the samples are stable for three months. Stored at 2–8 °C the samples are stable for a maximum of three hours and at room temperature (15–30 °C) only one hour. Please also refer to the information regarding quantification of zopiclone in section 7.3.

5.2 Sample preparation

5.2.1 Reconstitution of the lyophilised serum calibrators / controls

The ClinCal® Serum Calibrators and ClinChek® Serum Controls (order nos. MS6013 and MS6082, see section 2.1) are lyophilised and therefore need to be reconstituted before use. Information on reconstitution, analyte concentration, storage and stability is indicated in the respective product data sheets.

5.2.2 Reconstitution of the lyophilised Internal Standard IS

The ClinMass® Internal Standard IS (order no. MS9512) is lyophilised and has to be **reconstituted with Precipitation Reagent P (order no. MS9021)** prior to use.

Information on reconstitution, storage and stability is indicated in the product data sheet of the Internal Standard IS.

5.2.3 Work flow

Precipitation / Dotation with IS:	50 µl sample (calibrator, control, patient)	100 µl Internal Standard IS (reconstituted with Precipitant P)
	mix ⇩ (30 sec, vortex mixer)	
	centrifuge ⇩ (5 min, 10000 x g)	
LC-MS/MS Analysis:	Inject 1–10 µl* of the supernatant	

*Note 1:
The injection volume needs to be selected with respect to the sensitivity of the MS/MS system in use. If necessary the supernatant needs to be diluted with the Diluting Solution D (order no. MS9022).

Note 2:
Within one analytical series no different lots of a reagent should be used.

5.2.3.1 Precipitation / Dotation with IS

Pipette 50 µl of the sample (calibrator, control, patient) into the sample preparation vial (order no. MS9020). Add 100 µl of the Internal Standard IS (reconstituted with Precipitant P, see section 5.2.2) and mix for 30 sec on a vortex mixer. Centrifuge for 5 min at 10000 x g.

Transfer approx. 100 µl of the centrifuged supernatant into a suitable autosampler glass vial.

5.2.3.2 LC-MS/MS Analysis

Inject 1–10 µl of the sample into the LC-MS/MS system.

The injection volume needs to be selected with respect to the sensitivity of the MS/MS system in use. System-specific data for various MS/MS systems from different suppliers is available upon request at info@recipe.de. If necessary dilute the supernatant with Diluting Solution D (order no. MS9022).

5.2.3.3 Stability of the prepared samples

At room temperature (15–30 °C) the samples can be stored for at least three days. At temperatures between 2–8 °C the samples can be stored for at least 7 days and at temperatures below -18 °C for at least three months (multiple freeze-thaw cycles should be avoided).

Zopiclone:
Zopiclone has a shorter sample stability. The samples, stored at room temperature (15–30 °C), are stable for at least 12 hours and stored at 2–8 °C for at least 48 hours. Please also refer to section 7.3 for information regarding the quantification of zopiclone.

5.3 LC-MS/MS Analysis

Regardless of the analytical method the mass accuracy of the tandem mass spectrometer should be checked at regular intervals. A mass calibration may be required.

For information regarding the check-up of the MS/MS system, please refer to the instructions for use provided by the instrument manufacturer.

5.3.1 Optimisation of the tandem mass spectrometer

The optimisation of the MS/MS system comprises the optimisation of the ion source parameters as well as the compound-specific mass transitions. Five Optimisation Mixes (order nos. MS6015–MS6019), which contain all analytes, are available for this purpose.

The analyte composition of the respective optimisation mixes has been selected in a way so that the mass transitions will sufficiently differ from each other and thus provide an analyte-specific optimisation.

The optimisation mixes are lyophilised and therefore need to be reconstituted prior to use. Information regarding reconstitution, storage and stability is available in the respective product data sheets.

If necessary the optimisation mixes need to be diluted with Mobile Phase A with respect to the sensitivity of the MS/MS system in use. Device-specific data for the various LC-MS/MS systems by different suppliers is available upon request (info@recipe.de).

5.3.2 Equilibration of the analytical system and test run

Equilibrate the entire analytical system for at least 30 min before injecting samples.

At least three „blank-injections“ need to be carried out at the beginning of each analytical series (injection volume: 0 µl or injection of Mobile Phase A). This procedure facilitates reproducible analytical results from the first sample injection.

To perform a test run, repeatedly inject the optimisation mixes, until two consecutive chromatograms, comparable in retention times and peak areas, are obtained.

Depending on the required analytes (see product data sheets) the injection of the optimisation mixes can be performed subsequently or as a mixture of equal volumes.

Further dilution with Mobile Phase A of the reconstituted optimisation mixes may be necessary with respect to the sensitivity of the MS/MS system in use, see examples in Table 7.

Table 7. Dilution with Mobile Phase A (examples)

Target dilution	**Opti Mixes in use**	**Dilution with Mobile Phase A**
1:20	1	1:20
1:20	2*	1:10
1:20	5*	1:4

*Mixture of equal volumes

5.3.3 Calibration run

A ClinCal® 4-Level Serum Calibrator Set (Level 0–3, order no. MS6013) is available for calibration.

After reconstitution (see section 5.2.1) the calibrators need to be prepared as described for the patient samples (see section 5.2).

For each analytical series freshly pretreated calibrators have to be used.

5.3.4 Accuracy control

For the quality control of the analytical measurements ClinChek® Serum Controls in two different concentrations are available (Level I+II, order no. MS6082).

After reconstitution the controls need to be pretreated as described for the patient samples (see section 5.2).

For each analytical series freshly pretreated controls have to be used. In case of large analytical series we recommend injecting these controls additionally at the end of the series.

5.3.5 Example chromatogram

Figure 2 shows a chromatogram of the ClinCal® Serum Calibrator (order no. MS6013), Level 2, recorded with the LC system Agilent 1290 and the MS/MS system Agilent 6460:

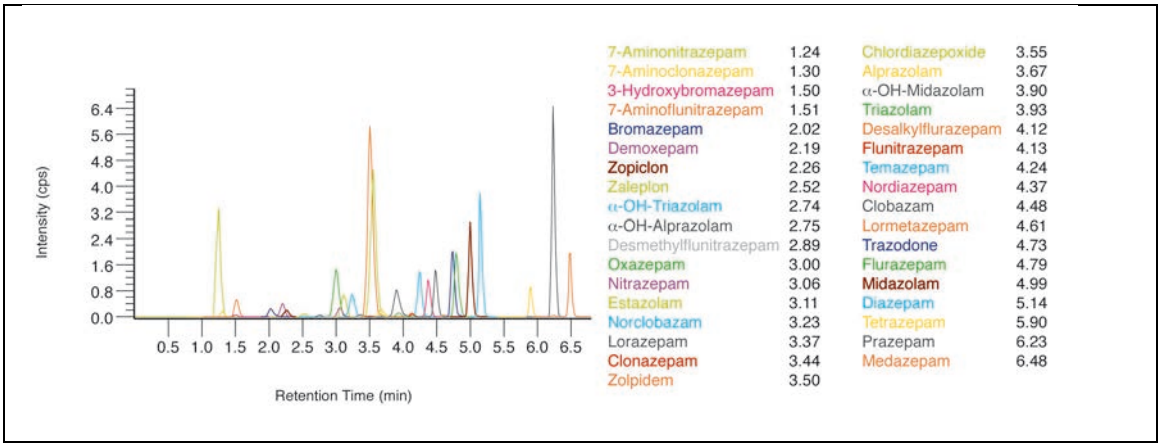


Figure 2. Chromatogram of the ClinCal® Serum Calibrator (order no. MS6013), Level 2

6 Evaluation

The analyte detection is performed via compound-specific mass transitions, see section 4.3.2.

The analyte concentration is calculated with the internal standard method via the peak areas.

Calibration curves are obtained from the calibrators by plotting the ratio *peak area „Analyte / internal standard“* against c*oncentration „Analyte*“.

The analyte concentrations in the samples and the controls are calculated from the calibration curves.

Please consult the software user manual of the MS/MS manufacturer in order to ensure correct evalution of the results.

For the conversion of the mass concentrations [µg/l] into molar concentrations [nmol/l] and vice versa, the analytical results should be multiplied with the factors listed in Table 8.

Table 8. Conversion factors

Analyte	**Molecular-weight [g/mol]**	**Conversion: nmol/l → µg/l**	**Conversion: µg/l → nmol/l**
3-OH-Bromazepam	332.15	0.332	3.011
7-Aminoclonazepam	285.73	0.286	3.500
7-Aminoflunitrazepam	283.31	0.283	3.530
7-Aminonitrazepam	251.29	0.251	3.979
α-OH-Alprazolam	324.70	0.325	3.080
α-OH-Midazolam	341.77	0.342	2.926
α-OH-Triazolam	359.20	0.359	2.784
Alprazolam	308.77	0.309	3.239
Bromazepam	316.15	0.316	3.163
Chlordiazepoxide	299.75	0.300	3.336
Clobazam	300.74	0.301	3.325
Clonazepam	315.72	0.316	3.167
Demoxepam	286.72	0.287	3.488
Desalkylflurazepam	288.71	0.289	3.464
Desmethylflunitrazepam	299.30	0.299	3.341
Diazepam	284.74	0.285	3.512
Estazolam	294.70	0.295	3.393
Flunitrazepam	313.29	0.313	3.192
Flurazepam	387.88	0.388	2.578
Lorazepam	321.16	0.321	3.114
Lormetazepam	335.19	0.335	2.983
Medazepam	270.76	0.271	3.693
Midazolam	325.77	0.326	3.070
Nitrazepam	281.27	0.281	3.555
Norclobazam	286.70	0.287	3.488
Nordiazepam	270.72	0.271	3.694
Oxazepam	286.72	0.287	3.488

Analyte	**Molecular-weight [g/mol]**	**Conversion: nmol/l → µg/l**	**Conversion: µg/l → nmol/l**
Prazepam	324.80	0.325	3.079
Temazepam	300.74	0.301	3.325
Tetrazepam	288.78	0.289	3.463
Trazodone	371.86	0.372	2.689
Triazolam	343.22	0.343	2.914
Zaleplon	305.33	0.305	3.275
Zolpidem	307.39	0.307	3.253
Zopiclone	388.81	0.389	2.572

7 Test data

7.1 Validation data

The validation data was established with the LC system Agilent 1290 and the MS/MS system Agilent 6460.

7.1.1 Linearity, detection limit and quantitation limit

Linearity, detection and lower quantitation limits are listed in Table 9.

Table 9. Linearity, detection- and lower quantitation limits (LOD, LLOQ)

Analyte	**Linearity [µg/l]**	**LOD [µg/l]**	**LLOQ [µg/l]**
3-OH-Bromazepam	1.00–1000	0.333	1.00
7-Aminoclonazepam	0.630–625	0.210	0.630
7-Aminoflunitrazepam	0.630–625	0.210	0.630
7-Aminonitrazepam	2.50–750	0.833	2.50
α-OH-Alprazolam	1.25–625	0.417	1.25
α-OH-Midazolam	2.50–1000	0.833	2.50
α-OH-Triazolam	2.00–500	0.667	2.00
Alprazolam	0.630–250	0.210	0.630
Bromazepam	3.75–1125	1.25	3.75
Chlordiazepoxide	10.0–10000	3.33	10.0
Clobazam	6.25–2500	2.08	6.25
Clonazepam	2.50–250	0.833	2.50
Demoxepam	25.0–7500	8.33	25.0
Desalkylflurazepam	2.50–1250	0.833	2.50
Desmethylflunitrazepam	0.630–250	0.210	0.630
Diazepam	12.5–2500	4.17	12.5
Estazolam	5.00–5000	1.67	5.00
Flunitrazepam	0.630–625	0.210	0.630
Flurazepam	1.00–1000	0.333	1.00
Lorazepam	2.50–2500	0.833	2.50
Lormetazepam	0.200–40.0	0.067	0.200
Medazepam	10.0–2000	3.33	10.0
Midazolam	3.75–1500	1.25	3.75
Nitrazepam	5.00–750	1.67	5.00
Norclobazam	75.0–15000	25.0	75.0
Nordiazepam	20.0–4000	6.67	20.0
Oxazepam	15.0–15000	5.00	15.0
Prazepam	10.0–3000	3.33	10.0
Temazepam	5.00–5000	1.67	5.00
Tetrazepam	5.00–2000	1.67	5.00
Trazodone	3.20–6400	1.07	3.20
Triazolam	1.00–500	0.333	1.00
Zaleplon	2.00–1000	0.667	2.00
Zolpidem	5.00–2000	1.67	5.00
Zopiclone	2.50–1000	0.833	2.50

7.1.2 Recovery

The recovery rate for all 35 benzodiazepines lies between 87.7–101 %.

7.1.3 Precision

The method intra- and interassay precisions were determined with samples in two different concentrations. The analyte concentrations were selected according to the respective therapeutic range and are contained in in Table 10 together with the precision results.

Table 10. Precision results

	Concentration [µg/l]		Intraassay Precision [%] (mean value)		Interassay Precision [%]	
Analyte	**Level**		**Level**		**Level**	
	I	**II**	**I**	**II**	**I**	**II**
3-OH-Bromazepam	50	166	3.9	3.9	8.4	7.2
7-Aminoclonazepam	15	50	1.6	1.4	3.8	6.8
7-Aminoflunitrazepam	15	50	1.5	1.2	4.1	5.7
7-Aminonitrazepam	60	200	1.6	1.5	4.9	5.6
α-OH-Alprazolam	15	50	3.7	4.2	8.6	7.5
α-OH-Midazolam	60	200	1.9	2.1	3.6	3.5
α-OH-Triazolam	12	40	5.4	5.4	5.9	5.2
Alprazolam	15	50	3.0	2.0	7.7	6.5
Bromazepam	90	300	3.5	3.9	6.0	6.4
Chlordiazepoxide	600	2000	2.8	1.3	2.8	2.6
Clobazam	150	500	2.4	2.1	4.7	5.0
Clonazepam	15	50	3.7	3.5	9.4	8.5
Demoxepam	600	2000	4.2	1.5	4.1	5.4
Desalkylflurazepam	30	100	3.8	2.9	7.8	7.1
Desmethylflunitrazepam	15	50	13.1	4.9	3.9	4.6
Diazepam	300	1000	1.7	1.3	4.7	6.4
Estazolam	120	400	2.3	2.8	2.3	6.2
Flunitrazepam	15	50	4.0	3.4	4.7	4.3
Flurazepam	24	80	1.1	1.8	3.3	4.3
Lorazepam	60	200	2.8	5.4	5.3	6.2
Lormetazepam	6	20	5.4	3.7	5.7	6.1
Medazepam	120	400	4.1	1.8	4.5	8.2
Midazolam	90	300	1.5	1.2	5.4	5.1
Nitrazepam	60	200	2.9	1.7	6.6	5.1
Norclobazam	900	3000	2.9	2.5	2.9	6.5
Nordiazepam	240	800	2.2	2.1	3.0	4.4
Oxazepam	360	1200	2.6	1.9	3.1	4.4
Prazepam	240	800	0.7	1.9	5.8	7.8
Temazepam	120	400	2.5	1.2	5.6	6.6
Tetrazepam	120	400	7.2	1.9	8.3	4.3
Trazodone	480	1600	1.0	2.6	2.4	8.3
Triazolam	12	40	5.5	3.9	5.9	6.3
Zaleplon	24	80	2.5	3.6	4.2	6.9

	Concentration [µg/l]		Intraassay Precision [%] (mean value)		Interassay Precision [%]	
Analyte	**Level**		**Level**		**Level**	
	I	**II**	**I**	**II**	**I**	**II**
Zolpidem	120	400	2.4	1.7	4.1	4.8
Zopiclone	24	80	3.6	6.3	12.4	10.3

7.2 Reference ranges

The following reference ranges are taken from the „Consensus Guidelines for Therapeutic Drug Monitoring in Neuropsychopharmacology: Update 2017" [6]. Values marked with * are taken from the data collection by Schulz et al. [7].

*Table 11. Reference ranges for benzodiazepines and metabolites according to [6], values marked with * according to [7]*

Analyt	Therapeutic Range [µg/l]	Laboratory Alert Level† [µg/l]
3-OH-Bromazepam	n.a.	n.a.
7-Aminoclonazepam	n.a.	n.a.
7-Aminoflunitrazepam	n.a.	n.a.
7-Aminonitrazepam	n.a.	n.a.
α-OH-Alprazolam	n.a.	n.a.
α-OH-Midazolam	n.a.	n.a.
α-OH-Triazolam	n.a.	n.a.
Alprazolam	5–50 20–40 (panic disorder)	100
Bromazepam	50–200	300
Chlordiazepoxide	400–3000	3500
Clobazam	30–300	500
Clonazepam	Anticonvulsant: 20–70 Anxiolytic: 4–80	Anticonvulsant: 80 Anxiolytic: 100
Demoxepam	*500–740	*Toxic: 1000
Desalkylflurazepam	n.a.	n.a.
Desmethylflunitrazepam	n.a.	n.a.
Diazepam plus N-Desmethyldiazepam (Nordiazepam)	100–2500	3000
Estazolam	*55–200	n.a.
Flunitrazepam	6–12 (sedation) 12–15 (sleep induction)	50
Flurazepam N-1-Desalkylflurazepam	0–4 (at 1–3 h) 10–22 (at 1–3 h) 75–165 (at 10 h after drug intake under steady state)	330
Lorazepam	30–100	300
Lormetazepam	2–10 (at 1.5 h)	100
Medazepam desmethyldiazepam, temazepam plus oxazepam	200–2500	3000
Midazolam	6–15 60–80 (at 1 h)	1000
Nitrazepam	30-100 (at0.5–2 h)	200

Analyt	**Therapeutic Range [µg/l]**	**Laboratory Alert Level† [µg/l]**
Norclobazam (Desmethylclobazam)	300–3000	5000
Nordiazepam (Nordazepam)	120–800	1500
Oxazepam	200–1500	2000
Prazepam	*200–700	*Toxic: 1000
Temazepam	600–1100 (at 1 h)	2000
Tetrazepam	*50–600 (–1000)	n.a.
Trazodone	700-1000	1200
Triazolam	2-20 (at 0.7–2 h)	40
Zaleplon	20–40 (at 1–2 h)	200
Zolpidem	80-160 (at 1–3 h)	320
Zopiclone	55–85 (at 1.5–2 h)	300

n.a.: not available;

† at values above the „Alert level“ the physician in charge should be informed immediately

The indicated reference ranges are taken from thoroughly selected and current scientific literature. Their actuality corresponds to the printing date of this document. Please note that these ranges do not reflect any recommendations by the manufacturer of this product, but may be used as a guideline for the assessment of the reference range by the clinical laboratory.

7.3 Note regarding the quantification of Zopiclone

Zopiclone is not stable in biological matrix, whereas the stability is strongly depending on pre-analytic factors such as storage duration or –temperature. The fast degradation of zopiclone at room temperature (15–30 °C) may lead to false results when measuring biological samples. For this reason it is recommended in scientific literature (e.g. [8]) to determine also the degradation product 2-Amino-5-chloropyridine (ACP; CAS 1072-98-6).

With this analytical method for benzodiazepines (order no. MS9500) it is also possible to analyse ACP. The following MRMs (ESI+) can be used:

Table 12. Retention time and mass transitions of the zopiclone metabolite ACP:

Analyte	**RT [min]**	**Quantifier MRM**		**Qualifier MRM**	
		Precursor [amu]	**Product [amu]**	**Precursor [amu]**	**Product [amu]**
2-Amino-5-chloropyridine (ACP)	2.8	129.0	112.0	129.0	76.2

When determining zopiclone, ACP should be integrated in the analytical method (it eluates at around the same time as 7-Aminoclonazepam). The absence of ACP in biological samples is a strong indication that the measured zopiclone concentration is reliable. In case however ACP is detected, the ACP concentration should be determined with the help of a calibration produced in matrix. The original zopiclone concentration (C0) of the sample is calculated as follows:

$$C0\ (Zopiclone)[\mu g/l] = (C\ (ACP)[\mu g/l]\ \times 3.02) +\ \ C\ (Zopiclone)\ [\mu g/l]$$

7.4 Interferences

7.4.1 Lorazepam

The qualifier transition of Lorazepam (*m/z* 321.0 → 275.0) shows a matrix interference. Due to this interference the transition can only be used at a concentration of above 250 µg/l.

The quantifier transition (*m/z* 321.0 → 229.0) is not affected by the interference.

7.4.2 Oxazepam

The qualifier transition of Oxazepam (*m/z* 287.2 → 104.1) is interfered by d_5-Nitrazepam. However, due to the constant d_5-Nitrazepam concentration, the transition can be used at a concentration of above 400 µg/l.

The quantifier transition (*m/z* 287.1 → 269.0) is not affected by the interference.

8 References

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9 Troubleshooting

Problem	Possible Cause	Corrective Measure
Retention times shifted	Defective HPLC pump	Check pumps
	Air within the system	Degas the mobile phases and flush HPLC
	Fluctuations of the flow rate	Check pumps
Interference signals	Injection system contaminated	• Rinse with methanol or inject 10 x mobile phase • Flushport: check solvent level • Clean/exchange injection needle and needle seat assembly
	Sample vials contaminated	Use new vials
	Vial septum contaminated	Use another septum
	Mobile phase contaminated	Change the mobile phases and flush the system
	Column(s) (guard / analytical column) contaminated	Change guard / analytical column
	Mass resolution too low	Optimise mass resolution
	System not properly installed	Check all connections
No signals	Defective injector	Check injector
	Defective HPLC pump	Check pump
	MS/MS system not ready for operation	Check MS/MS system
Decrease of sensitivity	Ion source contaminated	Clean ion source
	Mass spectrometer contaminated	Clean mass spectrometer
	Shift of mass calibration	Recalibrate MS/MS
	Mass resolution too high/low	Optimise mass resolution
	Injection valve leaking	Check injector

Problem	**Possible Cause**	**Corrective Measure**
Fluctuation of signal intensity	Spray unstable	Check spray needle capillary and clean if necessary
	Gas flow unstable	Check gas lines
No vacuum	Defective vacuum pumps	Check the pre- and high-vacuum pumps
	Vacuum system leaking	Check vacuum tubes and fittings
No gas supply	Defective nitrogen generator	Check nitrogen generator
	Defective compressor	Check compressor
	Gas bottle empty	Replace gas bottle
	Inlet gas pressures not within the specified range	Regulate the inlet gas pressures

10 Appendix: EC Declaration of Conformity

Declaration of Conformity

for in-vitro diagnostic medical devices, acc. to article 9 (1) of the directive 98/79/EG

The company

RECIPE Chemicals + Instruments GmbH
Dessauerstraße 3
80992 München / Germany

declares that the CE labelled products

ClinMass® TDM Platform (order no. MS9000) and
ClinMass® Add-On Set for Benzodiazepines in Serum / Plasma (order no. MS9500)

meet all applicable provisions of the directive on in-vitro diagnostic medical devices 98/79/EG. The conformity assessment was performed according to annex III. The technical documenation is held according to annex III no. 3.

Munich, 13.03.2018

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