Detection of benzodiazepines and barbiturates using a validated HS-SPME/GC-MS method

U. Kuepper, A. Birgel, F. Musshoff, B. Madea

Abstract

Aims: For a reliable diagnosis of brain death a possible influence of centrally acting drugs like benzodiazepines and barbiturates on the clinical picture has to be excluded beyond reasonable doubt. A confident detection of above mentioned pharmaceuticals with serum concentrations below the respective therapeutic level qualifies as a legit exclusion criterion.

Methods: An analytical method for the sensitive detection of benzodiazepines (diazepam=DIA, nordiazepam=NOR and midazolam=MID) as well as barbiturates (phenobarbital=PHE, thiopental=THI, pentobarbital=PEN and methohexital=MET) using headspace-solid phase microextraction (HS-SPME) in combination with gas chromatography-mass spectrometry (GC-MS) has been fully validated according to the GTFCh-guidelines [1,3]. To test for routine applicability the presented method is compared to our standard in-house high performance liquid chromatography-diode array detection (HPLC-DAD) method using reference samples.

Results: The method allows for the simultaneous detection of selected benzodiazepines and barbiturates and fully complies with the GTFCh-standards for precision and accuracy. The limits of detection and quantitation (GTFCh) were likewise satisfactory. Applicability was further proven in comparison to HPLC routine analysis.

Discussion: Due to the omission of a lengthy extraction the presented HS-SPME/GC-MS method is fast and easy, features which are especially desirable in brain death diagnosis. The detection method was fully validated and shown to comply with the current GTFCh-standards. Twin samples processed using either the novel HS-SPME/GC-MS method or the standard HPLC-DAD-procedure yielded comparable results. All of the above results prove that the presented method is applicable for routine analysis and represents a good alternative to conventional HPLC procedures.

1. Introduction

Brain death is defined as the irreversible shut-down of the cerebrum, cerebellum as well as the brain stem with the concomitant loss of all brain functions, thus marking the patients' death. Among others, the doubtless exclusion of any acute intoxications or the influence of centrally acting drugs on the clinical symptoms is a crucial prerequisite for the diagnosis of brain death. For this purpose, the modern toxicological analysis mostly uses hyphenated chromatographic techniques, e.g. HPLC-DAD, HPLC-MS or GC-MS. Analytical methods must allow for a confident detection of centrally acting pharmaceuticals even below their therapeutic serum concentrations to qualify as a legitimate

exclusion approach. In this context, the GTFCh recommends aiming for an LOQ of half the concentration of the lower therapeutic limit of each respective analyte to ensure the applicability of the method in brain death diagnostics [1], i.e. 0.5 µg/ml for both thiopental=THI and pentobarbital=PEN, 5 µg/ml for phenobarbital=PHE, 0.25 µg/ml for methohexital=MET, 0.02 µg/ml for midazolam=MID and 0.1 µg/ml for diazepam=DIA as well as nordiazepam=NOR.

It was our goal to fulfil the above requirements while omitting the usual time-consuming extraction procedures. Employing headspace-solid phase microextraction (HS-SPME) in combination with gas chromatographic-mass spectrometric (GC-MS) detection was considered a promising approach in the development a sensitive analytical method that is at the same time faster and easier than comparable GC-MS methods [4].

An analytical method for the sensitive detection of benzodiazepines (DIA, NOR and MID) as well as barbiturates (PHE, THI, PEN and MET) using headspace-solid phase microextraction (HS-SPME) in combination with gas chromatography-mass spectrometry (GC-MS) has been developed and optimized using an experimental design, i.e. central composite design, as previously described by our group: we were able to show that only a very specific combination of SPME-parameters enables the simultaneous detection of all analytes [2]. The resulting method was now fully validated according to the GTFCh-guidelines [1,3]. To test for routine applicability the presented method is compared to our standard in-house high performance liquid chromatography-diode array detection (HPLC-DAD) method using reference samples.

2. Materials and Methods

The monitored analytes consisted of the benzodiazepines diazepam, nordiazepam and midazolam (internal standard diazepam-d_5), as well as the barbiturates phenobarbital, thiopental, pentobarbital and methohexital (internal standard phenobarbital-d_5).

In order to evaluate method selectivity, blanks from different sources were analyzed as described below, but without adding any analyte or internal standard mix (n = 10). Further blanks were analysed to check for any interferences upon addition of internal standards (n = 2).

Concentration ranges (µg/ml) were chosen as follows for method validation: PHE: 2.5 – 50, PEN+THI: 0.5 – 10, MET: 0.2 – 4, DIA+NOR: 0.0625 – 1.25, MID: 0.025 – 0.5.
The concentration range comprised 10 almost equidistantly spaced calibrators, and was analysed 6-fold for confirmation of linearity. The results of the 6 lowest calibrators were used further for the calculation of LODs and LOQs.

Concentration (µg/ml) of *quality control* (QC) samples were:
QC low: PHE: 5, PEN+THI: 1, MET: 0.4, DIA+NOR: 0,125, MID: 0.05
QC medium: PHE: 20, PEN+THI: 4, MET: 1.6, DIA+NOR: 0.5, MID: 0.2
QC high: PHE: 40, PEN+THI: 8, MET: 3.2, DIA+NOR: 1, MID: 0.4
QC samples were analysed in duplicate on 8 different days.

During validation, *blank matrix* was pre-treated to yield the following *sample composition*:
- 1 ml blank serum
+ 50 µl calibrator/QC-mix
+ 40 µl internal standard-mix (final concentration: 5 µg/ml each)
+ 1 g NaCl
+ 1 ml 0.1 M citrate/0.2 M Na_2HPO_4 (58:42) buffer, pH 4.3

Analysis of *reference samples* was performed as follows:
- 1 ml of routine sample
+ 40 µl internal standard-mix
+ 1 g NaCl
+ 1 ml 0.1 M citrate/0.2 M Na_2HPO_4 (58:42) buffer, pH 4.3

SPME-extraction parameters were chosen as follows:
Extraction time: 30 min
Extraction temperature: 150°C

Experimental analysis was performed using the following *devices/settings*:
GC: HP 6890/Serie II Plus (Agilent)
MS-Detector: 5973 MSD (Agilent)
Autosampler: CTC CombiPal (Chromtech)
GC-column: HP-5MS (30m x 0.25mm x 0.25µm, Agilent)
SPME-fiber: 100 µm Polydimethylsiloxane coating (Supelco)
Flow: 1 ml/min Helium
Detection: in SIM mode (1 Quantifier and 2 Qualifiers each)
Temp. Prog.: 1 min @ 120°C, 10°C/min $\rightarrow$ 190°C (3 min hold),
             10°C/min $\rightarrow$ 250°C (4 min hold), 10°C/min $\rightarrow$ 300°C
             (6 min hold)

Validation data were calculated and evaluated using the VALISTAT-software [5].

3. Results and discussion

The presented method allows for the simultaneous detection of selected benzodiazepines and barbiturates. An exemplary total ion chromatogram (TICs) of a spiked serum sample is depicted in Fig. 1.
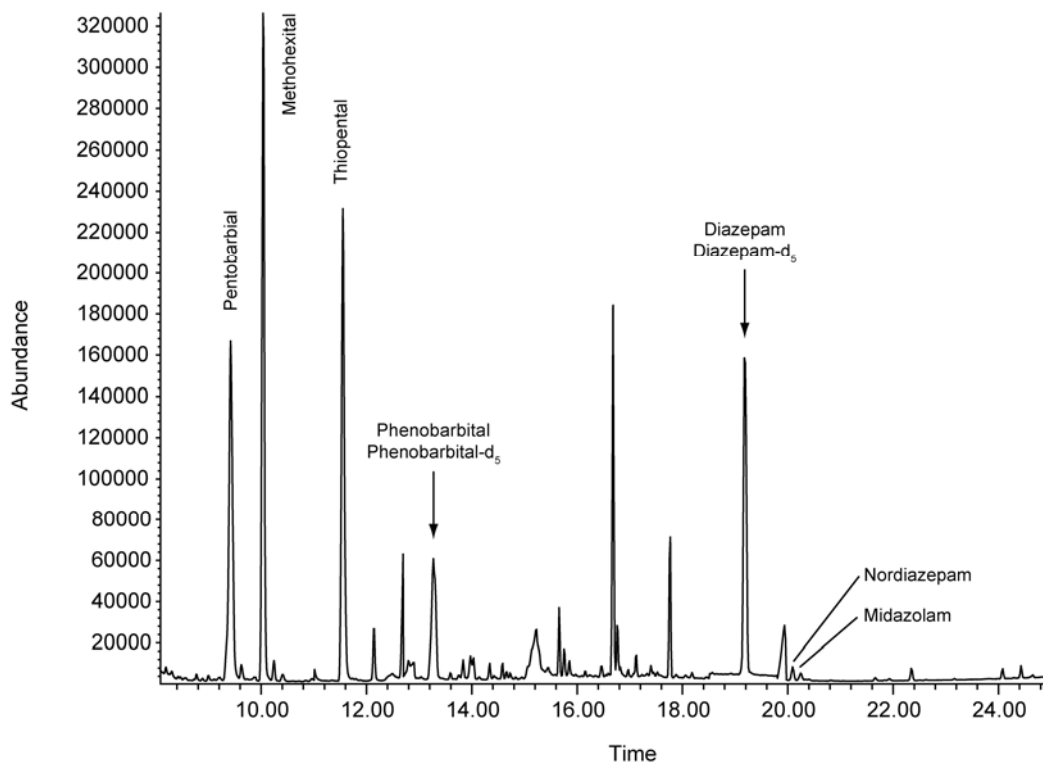
Fig. 1: Exemplary HS-SPME-GC/MS total ion chromatogram (TIC) of a serum sample with added calibrator mix "QC high"

Coefficients of correlations for the weighed calibration curves were consistently higher than 0.99 (Fig. 2; Tab. 1). The method was proven to fully comply with the GTFCh-standards for precision and accuracy, and the required limits of detection (LOD) and quantitation (LOQ) were likewise met (Tab. 1).

Table 1a: Summarized validation data of the presented HS-SPME-GC/MS method; m=slope, b=intercept, r=correlation coefficient

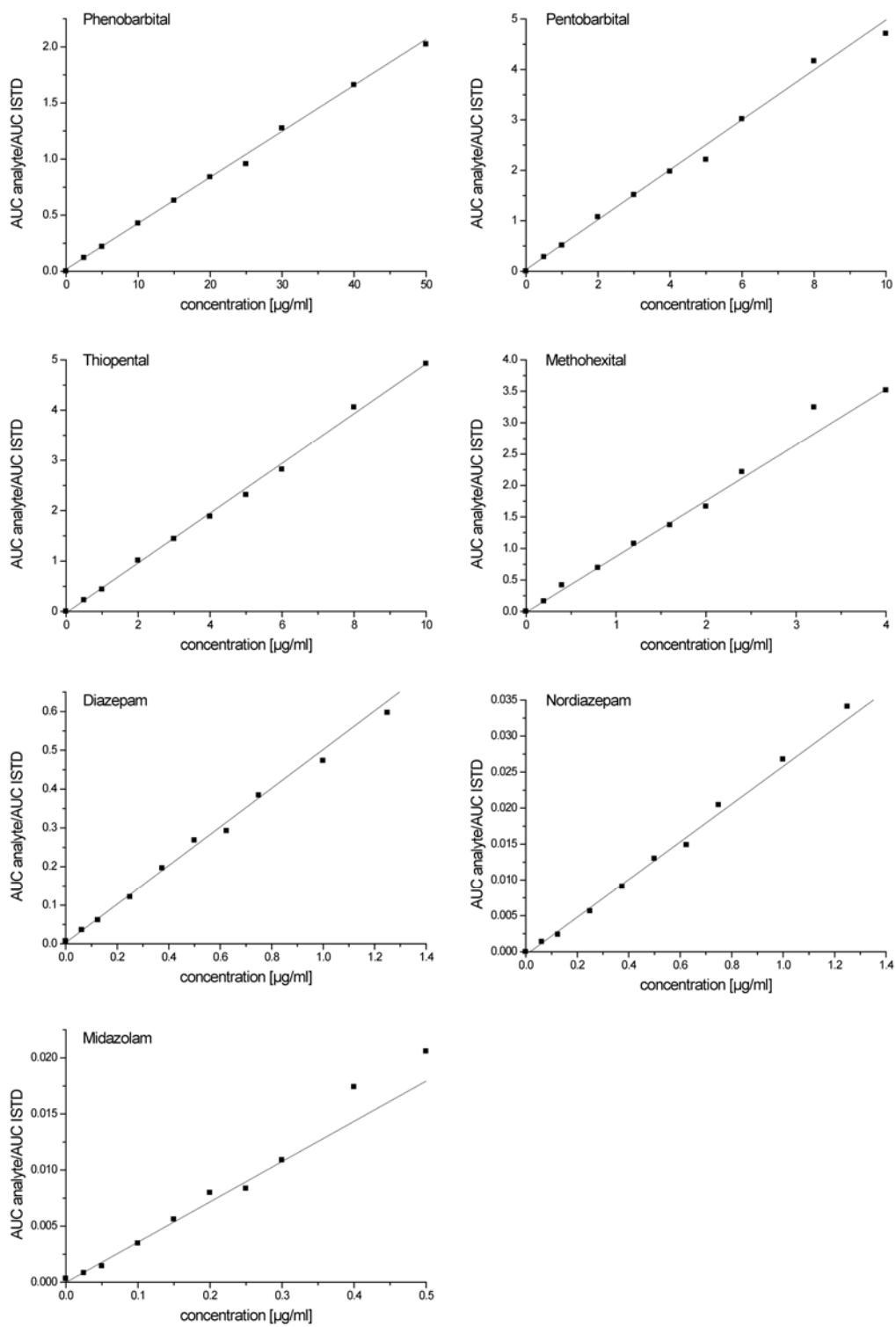| Analyte	m	b	r	LOD [µg/ml]	LOQ [µg/ml]
benzodiazepines					
diazepam	0.499	0.003	0.998	0.008	0.103
nordiazepam	0.026	-0.00044	0.998	0.015	0.097
midazolam	0.036	-0.00002	0.993	0.006	0.021
barbiturates					
phenobarbital	0.041	0.018	0.999	0.506	1.453
thiopental	0.495	-0.029	0.999	0.060	0.422
pentobarbital	0.495	0.033	0.996	0.063	0.442
methohexital	0.887	-0.015	0.993	0.058	0.146

Fig. 2: Weighed calibration curves for all validated analytes; AUC=area under the curve, ISTD=internal standard

Table 1b: Summarized validation data of the presented HS-SPME-GC/MS method; Reproducibility and accuracy.

| | QC low | | | QC medium | | | QC high | | |
Analyte	Intra-day [%]	Inter-day [%]	Accuracy [%]	Intra-day [%]	Inter-day [%]	Accuracy [%]	Intra-day [%]	Inter-day [%]	Accuracy [%]
Benzodiaz.									
diazepam	3.15	9.42	-1.91	3.22	12.28	-0.44	2.38	6.70	-2.50
nordiazepam	3.74	6.77	-6.53	4.23	10.65	-2.44	1.91	3.80	8.56
midazolam	6.01	8.42	-1.03	3.94	8.02	5.54	4.23	4.65	8.69
Barbitur.									
phenobarbital	4.60	5.30	3.71	1.16	3.90	0.65	1.38	3.73	5.49
thiopental	1.97	10.80	0.0	1.87	10.0	-2.74	2.91	4.76	8.14
pentobarbital	1.13	4.03	0.22	2.02	11.87	0.76	2.86	4.72	5.11
methohexital	5.98	11.49	3.19	1.85	5.87	3.09	3.04	5.04	8.17

Applicability was proven by analysis of authentic forensic samples (Fig. 3). A statistical evaluation of comparative analysis of twin samples processed using either the novel HS-SPME/GC-MS method or the standard HPLC-DAD-procedure is currently in progress (data not shown).
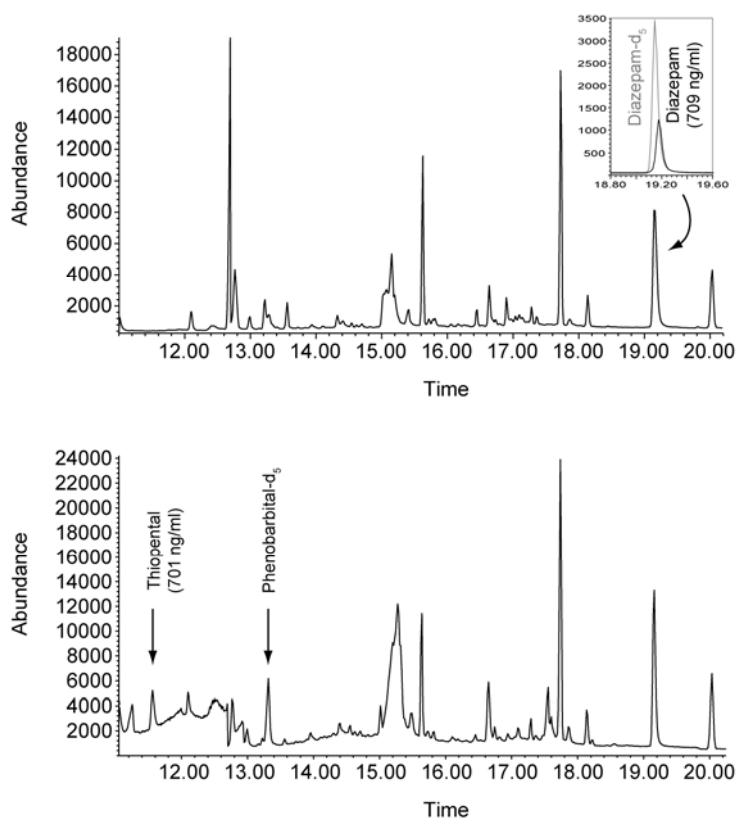

Fig. 3: Exemplary HS-SPME-GC/MS TICs of 2 authentic serum samples. Both benzodiazepines (DIA, top) and barbiturates (THI, bottom) are detectable. An extracted SIM chromatogram is shown for the differentiation of DIA (ion 256) and DIA-d5 (ion 261) (top).

Due to the omission of a lengthy extraction the presented HS-SPME/GC-MS method is faster and easier than comparable GC-MS methods [4], features which are especially desirable in brain death diagnosis.

All of the above results prove that the presented method is applicable for routine analysis and represents a valuable alternative to conventional HPLC procedures.

References

[1]   J. Hallbach, L. von Meyer und H.H. Maurer: *Empfehlungen des Arbeitskreises Klinische Toxikologie der Gesellschaft für Toxikologische und Forensische Chemie (GTFCh) für die toxikologische Analytik im Rahmen der Hirntod-Feststellung.* T + K (2002) 69 (3): 124

[2]   U. Kuepper, A. Birgel, F. Musshoff, B. Madea: *Anwendung eines experimentellen Versuchsdesigns zur Methodenoptimierung (HS-SPME-GC/MS) zum Nachweis von Benzodiazepinen und Barbituraten im Rahmen der Hirntoddiagnostik.* Vortrag 24 auf der 15. Frühjahrstagung (Nord) der DGRM in Dresden (9.-10.6.2006)

[3]   F.T. Peters, J. Hallbach und H.H. Maurer: *Empfehlungen der Gesellschaft für Toxikologische und Forensische Chemie (GTFCh) zur Validierung von Methoden für die toxikologische Analytik im Rahmen der Hirntod-Feststellung.* T + K (2004) 71 (2): 93

[4]   F.T. Peters, J. Jung, T. Kraemer, H.H. Maurer: *Fast, simple, and validated gas chromatographic-mass spectrometric assay for quantification of drugs relevant to diagnosis of brain death in human blood plasma samples.* Ther. Drug Monit. (2005) 27(3): 334

[5]   G. Schmitt, M. Herbold, F. Peters: *Methodenentwicklung im forensisch-toxikologischen Labor.* Arvecon GmbH, Walldorf, Germany, 2003

Uta Küpper
Institut für Rechtsmedizin
Universitätsklinikum Bonn
Stiftsplatz 12
D-53111 Bonn
E-Mail: Uta.Kuepper@ukb.uni-bonn.de

Alexander Birgel
Institut für Rechtsmedizin
Universitätsklinikum Bonn
Stiftsplatz 12
D-53111 Bonn
E-Mail: ABirgel78@aol.com

PD Dr. Frank Musshoff
Institut für Rechtsmedizin
Universitätsklinikum Bonn
Stiftsplatz 12
D-53111 Bonn
E-Mail: F.Musshoff@uni-bonn.de

Prof. Dr. Burkhard Madea
Institut für Rechtsmedizin
Universitätsklinikum Bonn
Stiftsplatz 12
D-53111 Bonn
E-Mail: B.Madea@uni-bonn.de