HPLC with Simultaneous UV and Reductive Electrochemical Detection at the Hanging Mercury Drop Electrode: A Highly Sensitive and Selective Tool for the Determination of Benzodiazepines in Forensic Samples

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Abstract

A versatile, sensitive, and selective high-performance liquid chromatographic (HPLC) procedure for the determination of common benzodiazepines and some of their most frequently occurring metabolites in forensic samples was developed and optimized with respect to effective and rapid sample preparation and high selectivity of the analytical assay. The optimized method includes liquid-liquid extraction of the drugs with chloroform followed by isocratic reversed-phase chromatography on a LiChrospher-100 RP-8ec column (150 x 4.6-mm i.d.) with a mobile phase consisting of 0.03 mol/L acetate buffer (pH 4.6)/acetonitrile (55:45, v/v). The use of dual-mode detection made up of UV-detection (250 nm) in series with reductive electrochemical detection (-1.4 V vs. Ag/AgCl) at the hanging mercury drop electrode permits the detection and quantitation of benzodiazepines even in degraded samples with higher selectivities than usually reached with conventional HPLC techniques. Depending on the actual benzodiazepine species, detection limits are in the range of 2.0 to 14.1 ng/mL. Mean recovery values of the drugs from blood range from 82 to 92%; within-day and day-to-day repeatabilities typically lie between 3 and 9%. Several case work examples demonstrate the high selectivity and remarkably low matrix sensitivity of the described assay.

Introduction

Benzodiazepines are drugs with hypnotic, anticonvulsant, and tranquilizing properties and are prescribed worldwide for the therapy of anxiety, sleep disorders, and convulsive attacks.

However, abuse and overdosage in combination with criminal offenses and road traffic accidents were the reasons that analysis of these drugs became of forensic interest in recent years.

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Note: The table and figure are not transcribed due to their complexity and the nature of the image. The abstract and introduction are provided in full.
According to their chemical structures, benzodiazepines are classified in three groups (1) (Figure 1): 1,4-benzodiazepines, diazolobenzodiazepines, and triazolobenzodiazepines, which can be further modified by different substituents at the benzodiazepine ring, giving rise to a number of different compounds with somewhat different pharmacological properties and chemical reactivity.

A number of analytical methods have already been applied for benzodiazepine analysis (2,3): immunoassays; gas chromatography (GC) with flame ionization detection (GC–FID), electron-capture detection (GC–ECD), or mass spectrometric detection (GC–MS) (2–5); and high-performance liquid chromatography (HPLC) with different detection modes, such as UV detection, fluorescence detection, MS (2–8), and electrochemical detection (8–14). However, though there are already a lot of methodologies and analytical data available, there is still a need for the development of new techniques and procedures. This is due to several reasons.

Identification and quantitation of benzodiazepines, especially in biological samples, are complicated by the fact that most of these drugs undergo extensive metabolism (15), leading to compounds that are often structurally similar to the parent drugs and pharmacologically active. Usually only minor amounts of the unchanged drugs are excreted. Some 1,4-benzodiazepines even have metabolic pathways in common, making it difficult to determine which kind of drug has been administered.

Additional problems arise from degradation and putrefaction of forensic samples (10,11), as well as from contamination of the sample by plasticizers or components of the elastomers used in storage bottles, septa, etc., which render the unambiguous identification and quantitation of benzodiazepines with conventional analytical techniques very difficult. Thus, the determination of benzodiazepines in biological samples requires analytical techniques that can detect small amounts of the parent drugs selectively in the presence of their metabolites without being vitiated by the presence of matrix components. Many existing methodologies, however, cannot cope with all of these demands.

Immunoassays, which are usually used for rapid screening in routine benzodiazepine analysis, lack selectivity and cannot discriminate between parent drugs and metabolites. Therefore, confirmation of screening techniques is required to assist in developing a definitive clinical diagnosis or to assess forensic implications. In many cases this is achieved by GC–ECD, GC–FID, or GC–MS. These methods are more sensitive and more specific than immunoassays and require more complex and expensive equipment and sometimes time-consuming derivatization procedures because benzodiazepines containing hydroxy groups adsorb readily on active sites of GC columns. Moreover, decomposition of thermolabile benzodiazepines, such as oxazepam and chlordiazepoxide, may occur during the GC process (4). This is the reason for the still increasing popularity of HPLC as a confirmation technique in the screening and quantitation of benzodiazepines. HPLC does not require derivatization or hydrolysis prior to analysis and is more suitable for the determination of thermolabile compounds. Single wavelength UV detection is very common and in many cases sufficiently selective for identification of the eluting peaks. In postmortem matrices, however, numerous compounds with similar retention behavior interfere in the chromatograms and lead to false-positive results.

Many different groups tried to solve these problems and to gain higher selectivity by applying more sophisticated sample preparation procedures, by improving the chromatographic separation and detection efficiency by gradient analysis (6), by using a multiwavelength detector (photodiode-array detection), or by attempting multi-detection approaches (9). However, very often these attempts proved to be very time-consuming or rather expensive.

We recently developed and optimized a procedure for the determination of benzodiazepines based on HPLC with UV detection in series with reductive electrochemical detection at the hanging mercury drop electrode (16). The reductive response of all benzodiazepines with the exception of clobazam (Figure 1) is due to the reduction of an azomethine group at about -1.4 V (vs. Ag/AgCl). Some benzodiazepines with more readily reduced nitro, amine oxide, or heterocyclic functions can be detected selectively at less negative potential. Effective deoxygenation of mobile phase and sample is essential for successful reductive electrochemical detection; otherwise, the reduction signal of dissolved oxygen will interfere in the chromatogram.

Because of the multi-detection approach, this methodology appeared to be especially promising for the determination of benzodiazepines in forensic samples. In this paper, the development of a rapid and precise sample preparation procedure and the successful application of the further optimized assay to several forensic case work examples is presented.

Experimental

Chemicals and reagents

All benzodiazepines were kindly provided by the Institute of Forensic Medicine (University of Innsbruck, Innsbruck, Austria). Stock solutions of benzodiazepine standards (1000 µg/mL) were prepared in methanol and stored in the dark at 4°C. The stock solution of internal standard contained 60 µg/mL n-methylclonazepam and was prepared freshly every week. Benzodiazepine standard solutions for recovery and quantitation experiments in real samples were prepared daily by further dilution of the stock solutions with methanol and mobile phase.

Acetonitrile, chloroform, methylene chloride, n-hexane, n-butylacetate, diethyl ether, cyclohexane (highest purity, p.a.), and buffer salts (highest purity, p.a.) were obtained from Fluka (Vienna, Austria); methanol (HPLC grade) was purchased from Merck (Vienna, Austria). Acetate (0.03 mol/L, pH 4.6), borate (0.1 mol/L, pH 8), and phosphate buffer (0.02 mol/L, pH 7) solutions were prepared by dissolving the appropriate amounts of phosphate, acetate, or borate salts in ultrapure water (Barnstead/Thermolyne, Dubuque, IA). The pH value of the final buffer solutions was controlled with a calibrated pH meter.

Plasma and whole blood needed for elaboration of the ex-
traction procedure and optimization of the assay were obtained from healthy volunteers.

**Apparatus**

The HPLC system consisted of an HPLC pump (Gynkotek, model 480), a Rheodyne injection valve (model 7010, Cotati, CA), a 20-μL loop (Rheodyne), and a UV absorbance detector (UV-975 Intelligent UV-vis detector, Jasco, Biolab GmbH, Vienna, Austria), which was operated at 250 nm. In series with the UV detector was an electrochemical detector (model 400, EG&G, Munich, Germany) in combination with a mercury LC-electrode (model LC-420, EG&G (PARC)). A conventional three-electrode configuration with a hanging mercury drop working electrode 303A (small drop size), a silver wire pseudo-reference electrode, and a platinum wire auxiliary electrode was used. Silver chloride was deposited on the silver wire electrode before use as the reference electrode. This coating had to be re-generated after several days if biological samples were analyzed. The detector was usually operated at a working potential held constant at –1400 mV vs. Ag/AgCl (decent current mode) before use as the reference electrode. This coating had to be re-generated after several days if biological samples were analyzed. The detector was usually operated at a working potential held constant at –1400 mV vs. Ag/AgCl (decent current mode) and in a current range of 10 to 20 nA (full scale).

**Table I. Detection Limits, Day-to-Day Precision (RSD), and Recoveries of Nine Benzodiazepines from Blood**

<table>
<thead>
<tr>
<th>Compound</th>
<th>Recovery (%) * and RSD within one week (%) +</th>
<th>Limit of detection (ng/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>25 ng/mL</td>
<td>50 ng/mL</td>
</tr>
<tr>
<td>EC</td>
<td></td>
<td></td>
</tr>
<tr>
<td>E: –1.4V (vs. Ag/AgCl)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Bromazepam</td>
<td>75 (–)</td>
<td>51 (7.0)</td>
</tr>
<tr>
<td>Oxazepam</td>
<td>78 (4.7)</td>
<td>56 (11.2)</td>
</tr>
<tr>
<td>Lorazepam</td>
<td>100 (5.0)</td>
<td>90 (9.7)</td>
</tr>
<tr>
<td>Nitrazepam a</td>
<td>93 (2.8)</td>
<td>99 (2.0)</td>
</tr>
<tr>
<td>Clonazepam a</td>
<td>101 (3.1)</td>
<td>98 (0.7)</td>
</tr>
<tr>
<td>Nordiazepam</td>
<td>88 (2.0)</td>
<td>88 (8.5)</td>
</tr>
<tr>
<td>Flunitrazepam b</td>
<td>103 (2.3)</td>
<td>101 (5.0)</td>
</tr>
<tr>
<td>Midazolam</td>
<td>62 (8.7)</td>
<td>75 (5.1)</td>
</tr>
<tr>
<td>Diazepam</td>
<td>78 (2.6)</td>
<td>79 (7.4)</td>
</tr>
<tr>
<td>UV λ = 250 nm</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Bromazepam</td>
<td>58 (7.9)</td>
<td>61 (18.2)</td>
</tr>
<tr>
<td>Oxazepam</td>
<td>74 (4.7)</td>
<td>70 (4.1)</td>
</tr>
<tr>
<td>Lorazepam</td>
<td>102 (5.1)</td>
<td>93 (1.4)</td>
</tr>
<tr>
<td>Nitrazepam a</td>
<td>99 (6.8)</td>
<td>100 (3.8)</td>
</tr>
<tr>
<td>Clonazepam a</td>
<td>95 (7.5)</td>
<td>100 (6.0)</td>
</tr>
<tr>
<td>Nordiazepam</td>
<td>109 (3.9)</td>
<td>96 (3.4)</td>
</tr>
<tr>
<td>Flunitrazepam b</td>
<td>100 (6.3)</td>
<td>92 (4.1)</td>
</tr>
<tr>
<td>Midazolam</td>
<td>74 (5.0)</td>
<td>83 (7.2)</td>
</tr>
<tr>
<td>Diazepam</td>
<td>83 (9.6)</td>
<td>98 (10.3)</td>
</tr>
</tbody>
</table>

* Recovery (accuracy) = \( \frac{X - \mu}{\sigma} \times 100 \) where \( X \) = mean of benzodiazepine concentration obtained in \( n \) independent measurements and \( \mu \) = "true" value (spiked benzodiazepine concentration).

+ Precision (relative standard deviation): RSD = \( \frac{S}{X} \times 100 \).

a LOQ values were calculated from the calibration curve (\( n = 4 \)) of each benzodiazepine in the range 2–50 ng/mL (see Experimental).

b Benzodiazepines containing a NO2-group moiety, such as nitrazepam, clonazepam, and flunitrazepam, were analyzed separately.

**Chromatographic conditions**

The separations were performed on a LiChrospher 100 RP-8ec column (150 x 4.6-mm i.d. with integrated precolumn, 5-μm particle size) at a constant column temperature of 9°C ± 1.0°C. Temperature control was achieved by cooling the column with ice-water mixtures. The mobile phase consisted of acetonitrile/acetate buffer (0.03 mol/L, pH 4.6) in a volume ratio of 45:55 (v/v) that was filtered through 0.45-μm cellulose acetate filters (Sartorius AG, Goettingen, Germany) before use. The flow rate was set to 1.0 mL/min.

The most efficient way to free the mobile phase from interfering oxygen was to reflux the eluent overnight (at a minimum eluent flow rate of 50 μL/min) while gently bubbling helium through the liquid. Deoxygenation was continued during the measurements. All polymer tubings were replaced by stainless steel capillaries in order to prevent back-diffusion of oxygen into the system. Oxygen in the sample was removed by gently purging the sample with argon for 10 min before injection. Evaporation and uncontrolled changes in the concentration of the sample were prevented by presaturation of the inert gas with mobile phase before passing it through the sample solution.

The deoxygenated sample was transferred into the sample loop by "suction loading" (removing about 100 μL of the sample by pulling at the injection syringe) instead of conventional direct injection ("pressure loading"). The injection volume was 20 μL.

**Extraction procedure**

In a plastic microcentrifuge tube 31.3 μL of n-methylchlorazepam standard solution (6 pg/mL), 300 μL borate buffer (0.1 mol/L, pH 8) and 600 μL chloroform were added to 500 μL of blood. After 5 min of agitation, centrifugation was performed at 13000 U/min for 10 min. Eighty percent (480 μL) of the organic layer was removed and evaporated to dryness using technical nitrogen gas and a Visidry extraction unit (Supelco, Vienna, Austria) that had been connected to a gas scrubber. The residue was dissolved in 300 μL of mobile phase and degassed as described previously. Twenty microilters of this solution was injected onto the column (by suction loading).

Plasma, urine samples, and smaller volumes of saliva (between 100 and 500 μL) were treated in the same manner as described for whole blood.

**Extraction recovery, calibration curves, and linearity**

During method development and selection of the best suitable extraction reagent, extraction recovery was evaluated by comparing the peak height of each benzodiazepine in spiked blood (plasma, urine) with the peak height obtained after direct injection of a known amount of standard. All further quantitative measurements using the optimized method.
as well as benzodiazepine determinations in real samples, such as hemolyzed human blood (plasma, urine, or saliva), were calibrated against a known amount of $n$-methylclonazepam, which had been added to the samples as internal standard prior to the extraction procedure. The extraction recovery was calculated from the peak-height ratios of the benzodiazepines relative to the internal standard compared with peak-height ratios obtained by standard mixtures, assuming that $n$-methylclonazepam exhibits the same extraction characteristics as the other benzodiazepines.

For calibration experiments and linearity tests, plasma, whole blood, and urine samples were spiked with known amounts of benzodiazepine standards in the range between 25 ng/mL and 200 ng/mL sample and extracted as described. Calibration curves were obtained by plotting the peak-height ratios of the benzodiazepines to the internal standard against the concentration of the benzodiazepine.

### Sensitivity and precision

The detection limits (LOD) listed in Table I for the individual benzodiazepines were calculated from the regression lines according to LOD = $b_0 + 3 \times S_b$ (17). The calculated intercept, $a$, was used as an estimate of $b_0$, the blank signal itself, and the standard deviation of the calibration line, $S_{bs}$, was used as an estimate of the standard deviation of the blank, $S_b$.

The precision of the analytical method (expressed as relative standard deviation, RSD) was determined by analyzing several samples of different concentrations within one day and on separate days over one week.

### Results and Discussion

#### Sample preparation

For real samples a sample pretreatment had to be elaborated that gave excellent recoveries and rapid results. First, precipitation of the proteins by addition of a ZnSO$_4$ solution to the sample, which had proven to be rapid and highly efficient in case of the determination of calcium antagonists in plasma, blood, and urine, was attempted (18,19). However, no satisfying recoveries and reproducibilities could be achieved for benzodiazepine-containing samples. Therefore, liquid–liquid extraction was chosen as an equally inexpensive and rapid alternative for sample pretreatment. The extraction process was optimized with respect to high recovery and reproducibility by variation of the type of extraction reagent, pH of the sample, and volume ratio of the extraction reagents relative to the sample volume. Initially, spiked plasma samples were extracted at a slightly alkaline pH (NaOH 0.05 mol/L), trying several organic solvents usually recommended in literature for the liquid–liquid extraction of benzodiazepines, such as hexane, cyclohexane, methylene chloride, diethyl ether, chloroform, and $n$-butylacetate. The best results were obtained with butylacetate and chloroform.

Figure 2 shows the extraction efficiency achieved with chloroform in comparison to butylacetate for plasma samples spiked with different amounts of benzodiazepines. Though butylacetate gave somewhat higher recoveries than chloroform, the latter was preferred for sample pretreatment because it was easier to remove when the extract was evaporated to dryness because of its higher volatility.

The influence of the pH of the plasma sample on the extraction recovery was studied in the pH range between 2 and 11 by adding appropriate amounts of 1M NaOH or 1M phosphoric acid to the plasma sample. The recoveries for bromazepam, lorazepam, nitrazepam, and midazolam were slightly better in alkaline media (82–98%), whereas clonazepam, nordiazepam, and diazepam were equally well extracted in acidic and alkaline media. Lower recoveries were obtained for all compounds at about pH 7 (72–93%), when the pH of the plasma was not modified. Only flunitrazepam showed significantly lower recovery values in strongly alkaline solutions (69%) than in slightly acidic media (94%). This might be explained by the fact that flunitrazepam is deprotonated in alkaline solutions and the resulting compound is less soluble in lipophilic extraction reagents than the parent benzodiazepine because of its acidity ($pK_a$ = 1.4). In moderately alkaline solutions (pH 8–9), about 80% of flunitrazepam and more than 82% of all other benzodiazepines could be recovered; therefore, all further extractions of plasma were carried out at this pH. Recovery values for all benzodiazepines were the highest if a volume ratio of 600 μL chloroform to 500 μL plasma was used.

As soon as recovery experiments were performed with whole blood, the pH adjustment had to be modified because the addition of 25 μL 1M NaOH to a 500-μL blood sample led to immediate coagulation. This could be prevented by adjusting the pH and simultaneously diluting the sample by the addition of borate buffer (pH 8) instead of 1M NaOH. The volume ratio of sample, buffer, and chloroform was varied with respect to optimized recovery. Ad-

![Figure 2. Comparison of the extraction recoveries of different concentrations of benzodiazepines with n-butyl acetate and chloroform from plasma. Results are the means of two independent replicates. Extraction procedure: 500 μL plasma + 19, 37.5, and 75 μL of a 2 μg/mL standard mixture + 25 μL 1M NaOH + 1 mL chloroform or butyl acetate.](https://academic.oup.com/jat/article-abstract/25/4/250/779268)
dition of 300 µL borate buffer (pH 8) and 600 µL chloroform to a 500-µL sample proved to be optimal for plasma recoveries higher than 86% for all benzodiazepines. Therefore, all samples including plasma, urine, and saliva were subsequently treated in the same manner as described (see Experimental).

Figure 3. Chromatogram of an extract from blood spiked with 10 benzodiazepines. Concentration of each benzodiazepine: 200 ng/mL (except IS: 500 ng/mL). Unidentified matrix peaks are indicated with an asterisk.

Table II. Retention Times and Interferences with other 1,4-Benzodiazepines (Flurazepam, Chlordiazepoxide) and Triazolo Benzodiazepines (Alprazolam, Triazolam, Clobazam)

<table>
<thead>
<tr>
<th>Benzodiazepine</th>
<th>Trade name</th>
<th>Retention time (min)</th>
<th>Interference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bromazepam</td>
<td>Lexotani®</td>
<td>3.38</td>
<td>3.51</td>
</tr>
<tr>
<td>Oxazepam</td>
<td>Adumbran®</td>
<td>4.45</td>
<td>4.56</td>
</tr>
<tr>
<td>Lorazepam</td>
<td>Tavor®</td>
<td>4.94</td>
<td>5.05</td>
</tr>
<tr>
<td>Nitrazepam</td>
<td>Mogadon®</td>
<td>5.48</td>
<td>5.58</td>
</tr>
<tr>
<td>Clonazepam</td>
<td>Ritovil®</td>
<td>6.27</td>
<td>6.41</td>
</tr>
<tr>
<td>Nordiazepam</td>
<td>Calmday®</td>
<td>6.65</td>
<td>6.75</td>
</tr>
<tr>
<td>Flunitrazepam</td>
<td>Rohypnol®</td>
<td>7.68</td>
<td>7.80</td>
</tr>
<tr>
<td>Midazolam</td>
<td>Dormicum®</td>
<td>8.36</td>
<td>8.47</td>
</tr>
<tr>
<td>n-Methylclonazepam (IS)</td>
<td></td>
<td>9.02</td>
<td>9.12</td>
</tr>
<tr>
<td>Diazepam</td>
<td>Valium®</td>
<td>10.01</td>
<td>10.14</td>
</tr>
<tr>
<td>Alprazolam</td>
<td>Alplax®</td>
<td>4.73</td>
<td>4.84 flurazepam</td>
</tr>
<tr>
<td>Chlordiazepoxide</td>
<td>Librium®</td>
<td>4.61</td>
<td>4.72 overlapping peak with oxazepam</td>
</tr>
<tr>
<td>Clobazam</td>
<td>Frisium®</td>
<td>7.95</td>
<td>*</td>
</tr>
<tr>
<td>Flurazepam</td>
<td>Dalmadorm®</td>
<td>4.73</td>
<td>4.84 alprazolam</td>
</tr>
<tr>
<td>Triazolam</td>
<td>Halcyon®</td>
<td>4.97</td>
<td>5.08 lorazepam</td>
</tr>
</tbody>
</table>

* Clobazam gives no signal with EC-detection, so it cannot be confused with flunitrazepam which comes next in the chromatogram.

Chromatographic conditions/selectivity

An assay based on isocratic reversed-phase HPLC with simultaneous UV and electrochemical detection that had been successfully optimized (16) for the simultaneous determination of the most common benzodiazepines, as well as their most frequently occurring metabolites, was applied throughout these investigations. The mobile phase had to be slightly modified as soon as biological samples were analyzed because matrix components led to more pronounced aging of the column. Some of the more lipophilic benzodiazepines were increasingly retained, probably because of stronger interaction of these compounds with developing active sites on the column, and as a consequence, the selectivity of the assay decreased. This problem could be solved completely by using acetate buffer (pH 4.6) instead of phosphate buffer (pH 6) in a mixture with acetonitrile as mobile phase. Only bromazepam gave tailing peaks at this pH, and if the column was used for a longer period of time, the limit of quantitation for this compound became higher and higher. In this case, the column had to be changed. Long-term drift in retention behavior during routine application was eliminated by comparing the retention times of the eluting peaks of unknown samples with those of standard mixtures that were analyzed immediately before and after the sample throughout each assay.

Figure 3 demonstrates the high selectivity of the additionally optimized assay, showing typical chromatograms obtained from an extract of blood that had been spiked with 10 benzodiazepines. If fresh blood was spiked with benzodiazepines for recovery experiments, an unidentified matrix peak (marked with an asterisk in Figure 3) with a retention time between bromazepam and oxazepam was detected with UV and EC detection. In most casework examples, however, when older and degraded blood samples had to be analyzed, no such peak was observed. In the case of oxazepam, sufficient deoxygenation of the sample was essential because the oxazepam peak would otherwise overlap with the reduction signal of oxygen. Interferences with other 1,4-benzodiazepines and some triazolo benzodiazepines were tested, and the results are shown in Table II. Among the compounds tested, alprazolam interferes with flurazepam, triazolam with lorazepam, and chlordiazepoxide tends to give an overlapping peak with oxazepam. Because of the application of two independent detection modes, clobazam can be distinguished unambiguously from flunitrazepam because the former gives no response with reductive electrochemical detection. However, in about 130 casework samples investigated so far, none of the interferences listed above were encountered. Thus, the elaborated assay can be considered sufficiently selective for most forensic problems.

Linearity, LODs, recovery, and precision

For all quantitation experiments, n-methylclonazepam (IS) was added to the samples as the internal standard (see Experimental). This compound was chosen because it is not a naturally occurring metabolite of the selected benzodiazepines but shows similar extraction and retention behavior and detection characteristics. Linearity was studied in the concentration range of 25 to 200 µg/L, and a good linear correlation between extracted benzodiazepine concentration and detector response (0.993 < r < 0.999) was found for all drugs.
The LODs were calculated from the standard deviations of the calibration graph measured in the range 2–50 ng/mL (see Experimental section) and were found to be between 2.0 and 14 ng/mL (Table I). Benzodiazepines with additional reducible nitro-groups gave the lowest detection limits if the compounds were determined in separate runs. LOD values generally might be lowered if the residue of the extract were dissolved in a very small volume of mobile phase. However, because degassing of the sample with inert gas and suction loading (instead of direct injection) proved to be very important for successful reductive electrochemical detection, only a minimum amount of 150 μL could be handled without problems and serious losses, giving the LOD values in Table I, which were sufficiently low for clinical and forensic analyses.

The accuracy of the developed assay (17,20) was assessed by performing recovery studies in whole blood. The results are summarized in Table I. Precision of the method was determined by carrying at least 3–4 samples of 4 concentrations of each drug (25–200 ng/mL) through the entire procedure in one analysis day. This was repeated on four days in one week. Relative standard deviations obtained within one day usually were between 4 and 9% for all compounds, concentrations, and detection modes. Only in the cases of midazolam, oxazepam, nordiazepam, and lorazepam at a concentration of 25 ng/mL, somewhat higher relative standard deviations between 11 and 13% were obtained with EC detection. Between-day precision data are listed in Table I.

Applications

Though at first sight single wavelength UV detection appears to be sufficiently selective (Figure 3) and less complicated in use than reductive electrochemical detection, it is certainly by far more sensitive to interferences by co-extracted matrix components of degraded samples. Identification of an eluting peak by UV detection, based on its retention time alone and the assumption that the peak is composed of only one single compound, may easily lead to false-positive results, which have to be verified by other analytical techniques. The described method was designed primarily to serve as a confirmation and quantitation technique, especially in cases in which benzodiazepines
had been detected by screening procedures such as radioimmunoassay, but could not be identified unambiguously and quantitated by GC-ECD, GC-MS, or HPLC-UV. This task was successfully accomplished by the elaborated assay, and the advantage of simultaneously using two different, independent, and highly sensitive detection modes is illustrated in the following examples.

Of the 130 forensic casework samples analyzed in this study, about 80% were taken from living persons involved in either a road traffic offense or drug abuse; the rest were taken postmortem. More than one benzodiazepine had been ingested in some cases, and very often exact assignment of the peaks to special benzodiazepine species was only possible with the help of reductive electrochemical detection. Diazepam, its major metabolites oxazepam and nordiazepam, midazolam, and flunitrazepam were among the most frequently encountered benzodiazepines. To a lesser extent, bromazepam and lorazepam were found in the materials to which the new technique was applied. A positive example from a road traffic case is shown in Figure 4A. Both detection modes indicate that diazepam has been digested by the person. The peak marked with an asterisk might have been mistaken for nordiazepam if only UV detection had been applied. However, because no signal corresponding to nordiazepam was observed with EC detection, the peak must be due to a matrix component.

Figures 4B, 5, and 6, show examples from drug abuse cases. In Figure 6, most of the ingested diazepam has already been transformed into the metabolites oxazepam and nordiazepam, and only traces of the parent drug are left. Unequivocal identification and quantitation of nordiazepam and flunitrazepam in the chromatograms in Figures 4B, 5, and 6 are almost impossible by UV detection because of coelution of irrelevant matrix components, whereas the chromatograms obtained with the reductive technique remain unaffected.

Without further modification, the elaborated assay was also applied to the analyses of urine samples (Figure 7). Urine samples are usually less frequently analyzed during forensic analyses because identification of the administered benzodiazepines is complicated because these drugs undergo extensive metabolism and only a minor amount of unchanged drug is excreted. On the other hand, in addition to the results obtained from blood samples, the identity of the ingested drugs often can be reconstructed from the pattern of the detected metabolites in urine, provided that the applied analytical technique is sufficiently sensitive and selective. Although lorazepam and nordiazepam could be monitored with both detection modes in Figure 7A, the remaining diazepam traces in this case could only be detected with UV detection because of the somewhat lower LOD and noise level reached with this detection mode. The chromatogram in Figure 7B shows an urine sample from a person who probably ingested diazepam, which has already been completely metabolized to nordiazepam. Again, reductive electrochemical detection is less hampered by matrix components than UV detection is.

Saliva and blood samples of highly drug addicted persons were analyzed in the same manner, and the chromatograms in Figure 8 demonstrate the extremely high sensitivity and versatility of the elaborated assay. Though blood concentrations in case of drug addiction are rather high, concentrations in saliva are negligibly small, and thus analysis of this medium requires a very sensitive analytical technique. None of the other techniques usually applied in benzodiazepine analysis was sensitive enough to detect benzodiazepine traces in saliva.

Conclusions

The assay presented in this paper is rapid, reliable, and highly versatile and sensitive. No derivatization procedure, only a simple extraction step, is required prior to analysis. Because of
the simultaneous application of two different and independent detection principles of comparable sensitivity, the developed methodology allows the reliable and selective determination of benzodiazepines in all kinds of biological samples encountered in forensic investigations, such as blood, urine, and even saliva.

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References


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