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Selective and sensitive assay for the determination of benzodiazepines by high-performance liquid chromatography with simultaneous ultraviolet and reductive electrochemical detection at the hanging mercury drop electrode

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Abstract

An isocratic chromatographic method for the simultaneous determination of 10 benzodiazepines is presented. The selectivity of the assay was optimized by variation of stationary phase, temperature, as well as ionic strength, composition and pH of the mobile phase and the dependence of the detector response on the applied potential was investigated. The best results with respect to resolution at moderate retention times were obtained with a mixture of 0.02 mol/l phosphate buffer (pH 6) and acetonitrile in a volume ratio of 55:45 (v/v) on a LiChrospher-100 RP-8ec column (150×4.6 mm I.D.). Considerable improvement of selectivity was achieved if the column temperature was kept constant at 12°C. Two detection modes were applied, UV detection at 250 nm, inserted upstream to the electrochemical detector, and reductive electrochemical detection at the hanging mercury drop electrode at −1.4 V (vs. Ag/AgCl), which proved to be especially sensitive in case of nitrophenyl-containing benzodiazepine species. The elaborated assay showed to be linear up to at least 2 mg/l for each compound. Detection limits generally were in the range of 6.5–123 ng/ml (130 pg–2.46 ng on-column, using a 20-μl loop) with relative standard deviations between 1.1 and 8.6% depending on the actual benzodiazepine investigated.

Keywords: Hanging mercury drop electrode; Benzodiazepines

1. Introduction

Due to their hypnotic, sedative and anticonvulsant effects benzodiazepines have become world-wide one of the most frequently prescribed and used anxiolytic drugs. However, the anti-anxietic and sedative effects of these drugs are also responsible for their emergence as substances of abuse in recent years. Thus rapid and reliable analytical assays are required not only to evaluate pharmacokinetics, bioavailability and clinical pharmacology, but also to detect and identify the drugs in toxicological and forensic samples involving road traffic accidents, drug overdoses and other criminal delicts.

In Scheme 1 the chemical structures of several common benzodiazepine species are summarized.

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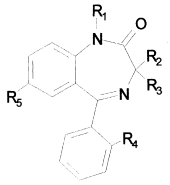
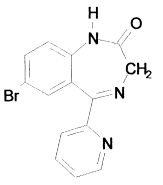
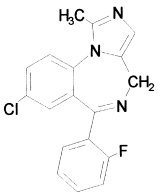


General benzodiazepine structure



Bromazepam (Broma) (Lexotanil®)



Midazolam (Mida) (Dormicum®)

benzodiazepine	trade name	R_1	R_2	R_3	R_4	R_5
clonazepam (Clona)	Ritrovil®	-H	-H	-H	-Cl	$-NO_2$
diazepam (Diaz)	Valium®	$-CH_3$	-H	-H	-H	-Cl
flunitrazepam (Flun)	Rohypnol®	$-CH_3$	-H	-H	-F	$-NO_2$
lorazepam (Lora)	Tavor®	-H	-H	-OH	-Cl	-Cl
nitrazepam (Nitra)	Mogadon®	-H	-H	-H	-H	$-NO_2$
nordiazepam (Nord)	Calmday®	-H	-H	-H	-H	-Cl
temazepam (Tema)	Restoril® Levanxol®	$-CH_3$	-H	-OH	-H	-Cl
oxazepam	Adumbran® Praxiten®	-H	-H	-OH	-H	-Cl
n-methylclonazepam (IS = internal standard)		$-CH_3$	-H	-H	-Cl	$-NO_2$

Scheme 1. Chemical structures of selected 1,4-benzodiazepines.

Benzodiazepines are generally characterized structurally by a 1,3-dihydro-5-aryl-2H-1,4-benzodiazepin-2-one ring system. A number of modifications in the benzodiazepine ring structure have resulted in a number of new compounds with similar pharmacological activities, but differences in lipophilicity, basicity and chemical reactivity. These differences are also responsible for the different response of benzodiazepines to analytical methods. Routine analysis of benzodiazepines comprises various techniques, such as radioimmunoassays for rapid screening, gas chromatography (GC) with electron-capture detection (ECD) and mass spectroscopy, high-performance liquid chromatography (HPLC) with ultraviolet (UV) detection, mass spectroscopy (MS) [1–5,10–12] and electrochemical detection (ED) [5–11]. Though compilations of analytical data covering detection and identification of various benzodiazepines are already available [1,2], there is still a need for the development of new analytical methods as well as the improvement of already existing procedures due to the remaining difficulties in the detection and quantification of these compounds and their metabolites in biological samples. GC methods in general (GC–ECD and GC–MS), in spite of a potential sensitive detection principle, suffer from the thermal instability and low volatility of many benzodiazepines, making tedious sample preparation steps necessary, such as derivatisation or acid hydrolysis of the parent benzodiazepines, which reduces the usefulness of these methods for the analysis of forensic samples [3].

In this report the development of a sensitive isocratic chromatographic procedure with simultaneous UV and reductive electrochemical detection is described. The assay was optimized with respect to enhanced selectivity concerning the determination of some benzodiazepine species, such as bromazepam, lorazepam and midazolam, which frequently caused problems during analysis of forensic samples with conventional analytical methods, such as GC–ECD, GC–MS and HPLC with UV detection.

2. Experimental

2.1. Chemicals and reagents

All benzodiazepines were kindly provided by the Institut für Gerichtsmedizin (University of Innsbruck, Innsbruck, Austria). Stock solutions of benzodiazepines (about $3 \cdot 10^{-3}$ *M*) in methanol were kept in the dark under refrigeration (4°C) to avoid decomposition. They were further diluted with mobile phase before use as benzodiazepine standard solutions.

Acetonitrile (puriss. analytical-reagent grade), methanol (HPLC-grade), 2-propanol (analytical-reagent grade) and buffer salts (puriss. analytical-reagent grade) were purchased from Fluka (Vienna, Austria). Buffer solutions were prepared by dissolving the appropriate amount of phosphate salts, $Na_3PO_4 \cdot 12H_2O$, $NaH_2PO_4 \cdot H_2O$, Na_2HPO_4 in ultrapure water with a conductivity of 18.2 MΩ (Barnstead/Thermolyne, Dubuque, IA, USA). The pH value of the buffer solutions was controlled with a calibrated pH meter. The mobile phase was filtered through 0.45-μm cellulose acetate filters (Sartorius, Germany) before use.

2.2. Apparatus

The HPLC system consisted of a HPLC pump (Gynkotek 480, Munich, Germany), a laboratory-made pulse damper, and a Rheodyne injection valve Model 7000 (Cotati, CA, USA) with a 20-μl sample loop. In series with the UV–Vis detector (Jasco, UV-975, Biolab, Vienna, Austria) was an electrochemical detection system (EG&G, Model 400, Munich, Germany). Reductive detection was performed on a hanging mercury drop electrode (HMDE) (EG&G, Model 420). Recording of the chromatograms as well as quantitative data analysis was accomplished on a personal computer-based data system (GynkoSoft, Version 5.5, Gynkotek).

2.3. Chromatographic conditions

Chromatography was carried out on Nucleosil RP-4ec (250×4 mm I.D.) and LiChrospher RP-8ec (250×4 mm I.D.; 150×4.6 mm I.D.; 60×4.6 mm I.D.) columns with integrated guard columns (Knauer, Berlin, Germany). In order to remove oxygen, the mobile phase was refluxed at 60–80°C and helium gas was gently bubbled through the liquid. Deoxygenation of the mobile phase was performed overnight (at a minimum eluent flow-rate of 50 μl/min) and continued during the measurements. All polymer tubings were replaced by stainless steel capillaries. Oxygen in the sample was removed by gently purging the sample with argon for 10 min before injection. Evaporation or 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 flow-rate was 1.0 ml/min and the columns as well as the detection units were kept at ambient temperature unless otherwise stated. Temperature control of the column was in the range of ±0.5°C. In the temperature region between 0 and 20°C temperature control was achieved by using ice–water mixtures. For higher temperatures (>20 to 60°C) the built-in column oven of the Antec Decade electrochemical detector was applied. The UV–Vis detector was operated at 250 nm whereas the electrochemical detector was used in decent current mode at potentials between −600 to −1500 mV vs. Ag/AgCl (pseudo-reference electrode).

3. Results and discussion

3.1. Optimization of the chromatographic system

Many different combinations of stationary and mobile phases have already been reported for chromatographic benzodiazepine analysis [1,2]. We selected a LiChrospher-100 RP-8ec (150×4.6 mm I.D.) column as stationary phase, which after some rapid screening tests promised to give the best compromise with respect to resolution and migration time and which had already been applied successfully to the analysis of other drugs of similar polarity [13]. The selectivity of the separation was further manipulated by variation of the mobile phase. Initially methanol was tested as organic modifier, because it is one of the most frequently used solvents in benzodiazepine analysis. Phosphate buffer (pH 6, $I=0.02$ M) was chosen as supporting electrolyte, in order to achieve sufficient conductivity for electrochemical detection. However, with mixtures of methanol and phosphate buffer bad resolution, long retention times and bad peak symmetry was obtained for some benzodiazepines which could neither be improved by variation of the amount of methanol, nor, in contrast to literature reports [5,6] by addition of small amounts of 1-propanol. Much better results with respect to high resolution and comparatively short analysis time were obtained if methanol was substituted by acetonitrile. In Fig. 1 three typical chromatograms are presented, which demonstrate clearly the influence of the type of organic modifier in the mobile phase on the benzodiazepine separation. Especially the retention times of *n*-methylclonazepam, midazolam and flunitrazepam were very sensitive to solvent changes. With a mixture of phosphate buffer and acetonitrile (Fig. 1a) in a ratio of 55:45 (v/v) almost all compounds, except lorazepam, were almost baseline resolved within 10 min. The remaining problem of co-elution in case of lorazepam and nitrazepam could not be

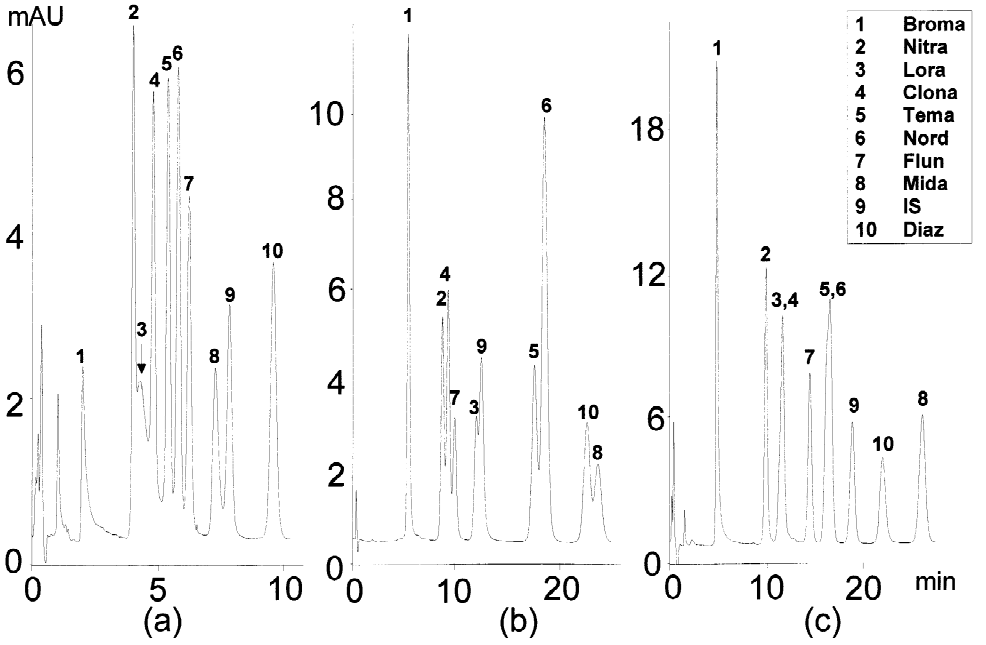


Fig. 1. Effect of the composition of the mobile phase on the elution of 1,4-benzodiazepines. Mobile phase (a) 0.02 *M* phosphate, pH 6.0–acetonitrile (55:45, v/v), (b) 0.02 *M* phosphate, pH 6.0–MeOH (45:55, v/v), (c) 0.02 *M* phosphate, pH 6.0–acetonitrile–MeOH (60:30:10, v/v/v); injection of (a) 0.05 μg (midazolam 0.07 μg) (b) 0.2 nmol (0.3 nmol bromazepam and 0.47 nmol nordiazepam) and (c) 0.1 μg of each drug. Column: LiChrospher-100 RP-8ec (150×4.6 mm I.D.).

solved by variation of the phosphate buffer–acetonitrile ratio between 60:40 and 55:45 (v/v) (Fig. 2), thus the buffer–acetonitrile ratio of 55:45 (v/v) was chosen as the most adequate solvent composition in all further optimization experiments.

The influence of ionic strength and pH of the buffer solution was also considered for optimization of resolution. However, increasing the ionic strength from 0.005 to 0.1 *M* led to co-elution of midazolam and flunitrazepam as well as temazepam and clonazepam, whereas the resolution of lorazepam was only slightly improved (Fig. 3). Best separation of all compounds, except lorazepam, was obtained with an electrolyte concentration of 0.02 *M*.

When the pH of the buffer solution was decreased, the k' values of the drugs decreased only slightly and the elution order remained more or less constant, with one exception. The midazolam peak was shifted drastically to lower k' values (see peak 8 in Fig. 4) at lower pH values (pH<4). Obviously at lower pH values midazolam is transformed into a protonated ionic compound, which is less retained on the reversed-phase column than the neutral parent compound. This observation can be explained by the fact that most benzodiazepines contain only a weakly basic imine moiety and hence have pK_a values ranging from 1.4 to 3.4. Differences in basicity (Table 1) and chemical reactivity of the compounds arise from additional substituents R_1–R_5 (Scheme 1). In case of midazolam and other 1,2-heterocyclic fused benzodiazepines, ring opening by imine hydrolysis occurs readily in the presence of aqueous acids. The resulting primary amine group of the hydrolysis product is a comparatively strong base compared to the cyclic parent benzodiazepine. This compound is protonated at acidic pH and as a consequence elutes faster than the other less basic drugs. Best separation of the benzodiazepines was observed when the pH of the mobile phase was kept between 6 and 7, but the problem of co-elution of lorazepam and nitrazepam could not be solved by variation of the mobile phase.

The most striking effect on the chromatographic separation, however, was observed, when the column

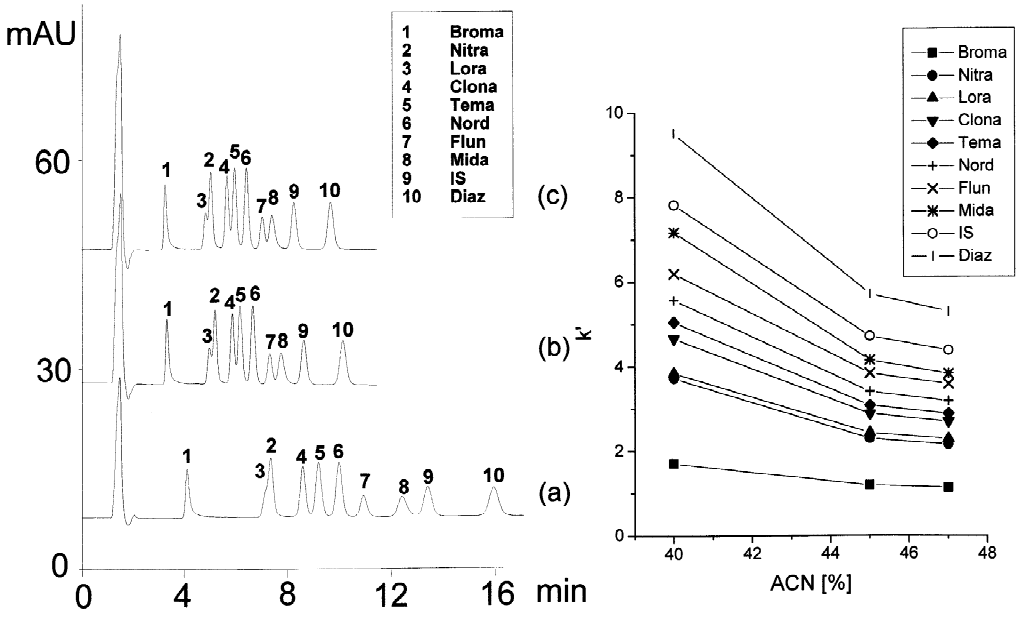


Fig. 2. Effect of % acetonitrile in the mobile phase on the separation. (Left) Chromatograms and (right) corresponding k' values of 10 benzodiazepines. Mobile phase: 0.02 *M* phosphate, pH 6.0+6 m*M* triethylamine–acetonitrile (a) 60:40 (v/v), (b) 55:45 (v/v), (c) 53:47 (v/v). Injection of 0.2 nmol of each drug. Column: LiChrospher-100 RP 8ec (150×4.6 mm I.D.).

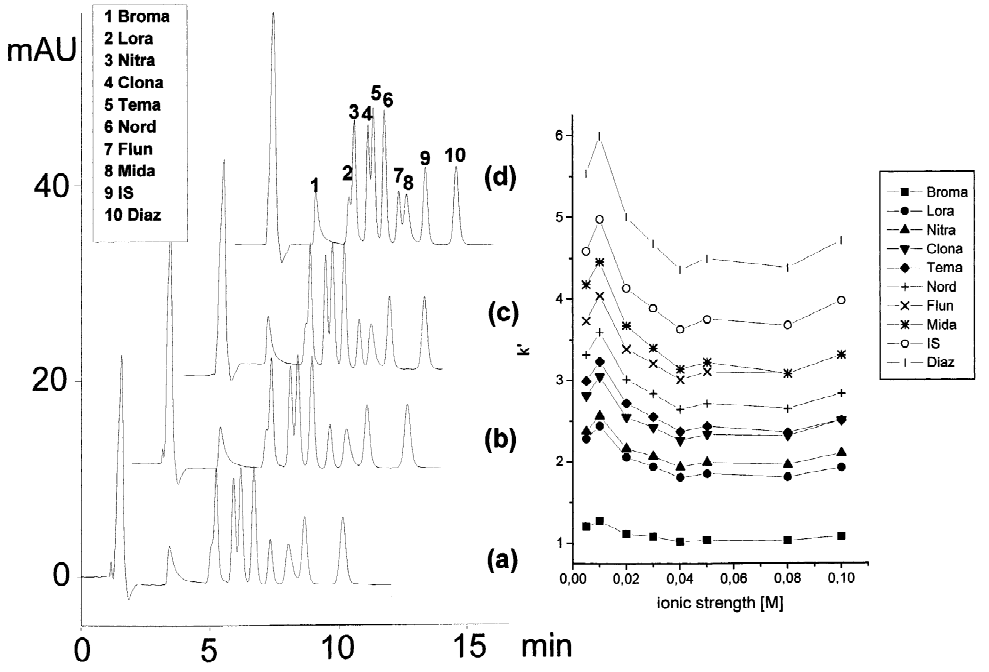


Fig. 3. Effect of the ionic strength of the buffer on the retention of benzodiazepines. (Left) Chromatograms and (right) corresponding k' values. Mobile phase: (a) 0.005 *M*, (b) 0.01 *M*, (c) 0.02 *M* and (d) 0.03 *M* phosphate, pH 6.0–acetonitrile (55:45, v/v). Injection of 0.2 nmol of each compound.

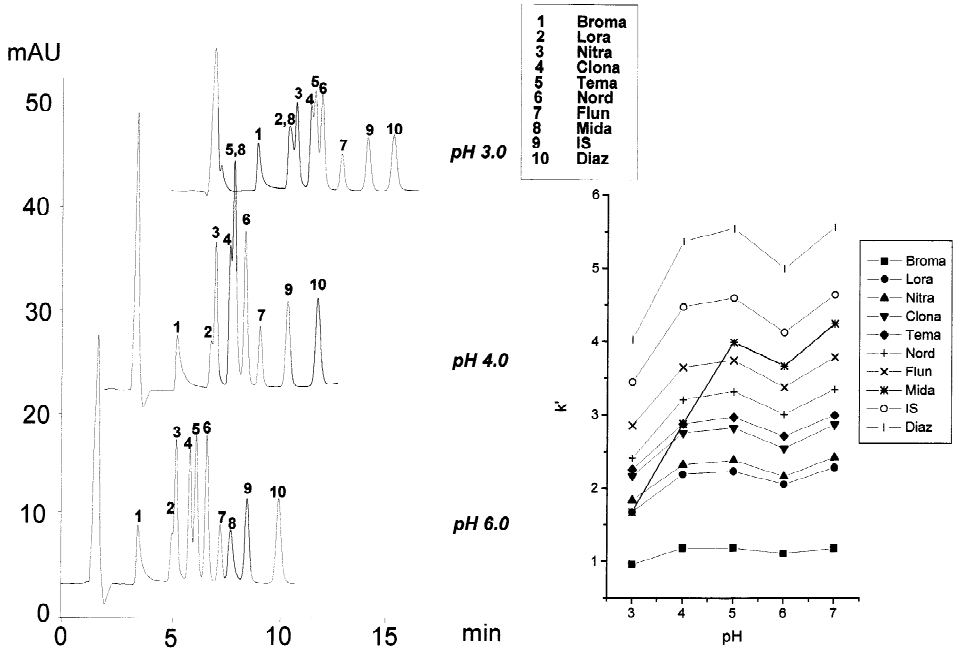


Fig. 4. Effect of the pH of the mobile phase on the elution of benzodiazepines. (Left) Chromatograms and (right) corresponding k' values. Mobile phase: 0.02 *M* phosphate, pH 3.0–7.0 (acetate, pH 5.0)–acetonitrile (55:45, v/v). Injection of 0.2 nmol of each drug.

temperature was varied. Though temperature effects, in combination with mobile phase variations, have been treated theoretically with respect to evaluation of retention mechanisms by one group [12], in almost all of the numerous papers about chromatographic benzodiazepine determinations published by other groups the importance of temperature variations is not taken into account when changes in the experimental parameters are discussed. However, it was surprising to observe to what extent resolution and elution order of benzodiazepines could be influenced by temperature variation. As shown in Fig. 5 the decrease of the k' values with increasing column temperature is more pronounced in case of *n*-methylclonazepam (I.S.), flunitrazepam, clonazepam and nitrazepam leading at ambient temperatures to co-elution of these compounds with other benzodiazepines, which are less sensitive to temperature changes. The separation could be considerably improved if the column temperature was decreased below room temperature. Selective determination of all 10 benzodiazepines was achieved within 12 min when the column temperature was kept constant at 12°C.

Table 1
pK_a values of selected benzodiazepines

Benzodiazepine	pK_a value	Ref.
Bromazepam	2.9	[19]
Lorazepam	1.3; 11.5	[20]
Nitrazepam	2.98–3.82; 10.55–11.51	[20–22]
Clonazepam	1.5; 10.2	[22]
Nordiazepam	11.82	[20]
Flunitrazepam	1.4	[23]
Diazepam	3.4	[19]
Temazepam	1.46	[24]
Midazolam	5.91–7.08	[21]

3.2. Optimization of electrochemical detection

Thus, by optimizing the chromatographic conditions a selective assay was elaborated which permits the simultaneous determination of 10 benzodiazepines in reasonable short analysis time. A further increase in selectivity and sensitivity was expected if a combination of two different detection modes was applied instead of using only UV detection. We decided to use reductive electrochemical

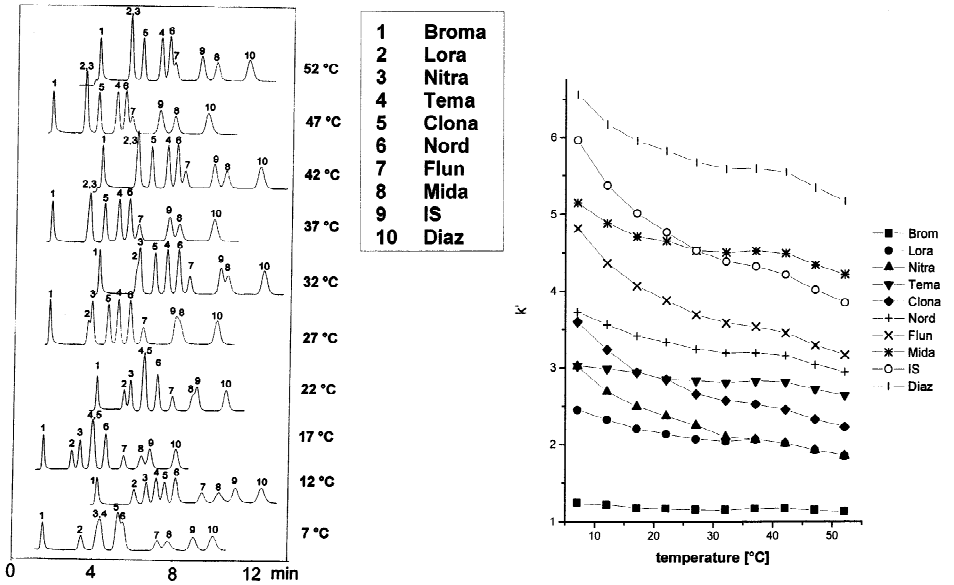


Fig. 5. Effect of column temperature on the separation efficiency. (Left) Chromatograms and (right) corresponding k' values. Mobile phase: 0.02 *M* phosphate, pH 6.0+6 m*M* triethylamine–acetonitrile (45:55, v/v). Injection of 0.2 nmol of each compound.

detection at the HMDE, because during earlier studies this detection mode had proven to be less sensitive to matrix components than other methods [13,14]. Electrode fouling, which usually is one of the most tedious problems whenever stationary electrodes are applied during long-term measurements, is easily circumvented by producing a new electrode surface by mercury drop dislodge. Moreover, the high overpotential of hydrogen reduction on mercury renders this electrode material best suited for reductions at fairly negative potential even in slightly acidic media. Most of the published work on liquid chromatography of benzodiazepines has employed UV detection, whereas reductive electrochemical detection of benzodiazepines especially on the HMDE is still rarely documented in literature [5–7,9]. This might be due to the fact that this detection mode demands more experimental skill than other methods. Oxygen has to be removed more or less completely from mobile phase and sample solution, because otherwise reduction of oxygen traces will lead to huge interfering signals. To a lesser extent problems in handling the hanging mercury drop electrode, irreproducibility of the working electrode area or higher noise levels were reported to be responsible for the less frequent use of this detection system. However as soon as these problems are solved, reductive detection at the HMDE proves to be a really valuable tool for detection in HPLC, improving both sensitivity and selectivity especially in case of analysis of complex biological samples [13,14].

Though UV detection was used throughout optimization of the chromatographic separation, the experimental conditions elaborated above were equally suitable for the use of electrochemical detection. Due to effective deoxygenation of the solvents, the low concentration and moderate pH (6–7) of the buffer salt in the mobile phase, the background current was low, providing nonetheless sufficiently high conductivity for the electrode reaction to take place. Further improvement concerning background current and noise was achieved when the reference electrode consisting of a silver/silver chloride wire in a solution of saturated KCl/AgCl, contained in a glass jacketed sleeve with a Vycor frit (attached via heat shrinkable tubing), was replaced by a silver wire pseudo-reference electrode on which silver chloride had been deposited electrochemically prior to use [7,8,25–30]. With this reference electrode system the measurements became more reproducible and sensitive, because the background current was markedly

reduced to allow measurements at 10 nA full scale with a peak-to-peak variation ($N_{p\leftrightarrow p}$; ''noise'') of about 3 pA. The potential of this reference electrode remained constant for several weeks, then the silver chloride coating had to be regenerated.

The redox mechanisms of benzodiazepines had been previously investigated by means other than HPLC [15]. However, optimization of electrochemical detection comprised evaluation of the reduction potential fitting best for the detection of benzodiazepines under the experimental conditions elaborated out so far for optimized chromatographic separation. For this purpose hydrodynamic voltammograms were recorded by stepwise variation of the applied potential. The dependence of the detection signal on potential is demonstrated in Fig. 6. All benzodiazepines are electroactive and can be determined electrochemically by reduction of the imino-group in the diazepine ring in the range between −1.2 and −1.4 V. For a general screening of benzodiazepines a working potential of −1.4 V (vs. Ag/AgCl) appeared to be appropriate. In case of nitrazepam, flunitrazepam, clonazepam and *n*-methylclonazepam, the presence of a nitro-phenyl group, which is reduced in the potential range between −600 to −800 mV, yields the possibility to detect those substances already at lower potential (at about −800 mV). This is important whenever correct assignment of the chromatographic signal to a benzodiazepine species appears to be difficult (e.g., in case of co-elution or overlapping peaks). Additional informations about structure and identity of the drugs in the sample can be obtained from electrochemical detection, simply by varying the detector potential. Thus, the simultaneous use of UV and electrochemical detection provides the possibility of higher selectivity.

3.3. Linearity, limit of detection and precision

In order to determine whether the elaborated assay responded linearly to concentration changes of the sample, benzodiazepine standard mixtures with different concentrations ($n=5$) were measured repeatedly ($m=3$) using the optimized chromatographic procedure described above, with a UV detector (at 250 nm) and an electrochemical detector (at −1.4 V) in series. The results are summarized in Table 2. All

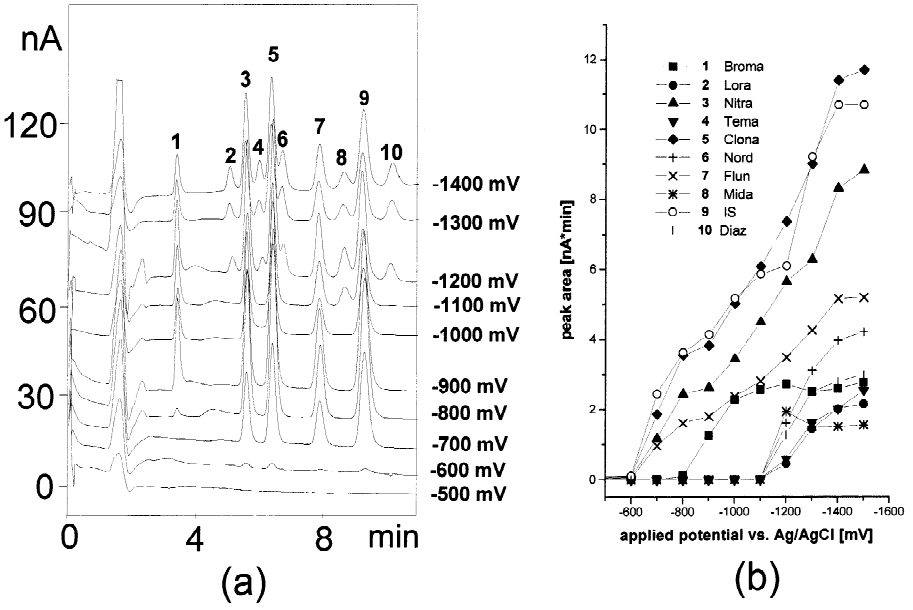


Fig. 6. Dependence of the detector signal on the applied potential. Chromatograms at selected potentials (a) and hydrodynamic voltammograms (b). Reductive mode [working electrode: hanging mercury drop electrode (HMDE), reference electrode: Ag/AgCl]. Mobile phase: 0.02 *M* phosphate, pH 6.0+6 m*M* triethylamine–acetonitrile (55:45, v/v); injection of 0.2 nmol of each drug.

Table 2
Linear regression and correlation parameters for the calibration curves of selected benzodiazepines

Benzodiazepine	Slope (b)/[S_b] [nA/(μg/ml)]	Intercept (=peak height, a)/[S_a] [nA]	Correlation coefficient	LOD[a] (ng/ml)	RSD (%)
ED					
Bromazepam	1.5181 [0.015]	0.0145 [0.016]	0.9997	49	3.2
Lorazepam	1.3143 [0.026]	−0.0590 [0.026]	0.9988	94	6.7
Nitrazepam	5.0421 [0.069]	−0.0892 [0.071]	0.9994	66	4.6
Clonazepam	6.4714 [0.021]	−0.0597 [0.022]	0.9999	6	1.2
Nordiazepam	2.3468 [0.008]	−0.0141 [0.009]	0.9999	18	1.3
Flunitrazepam	5.8037 [0.042]	−0.0625 [0.044]	0.9998	34	2.4
Midazolam	0.66151 [0.014]	−0.0009 [0.014]	0.9985	103	7.3
Diazepam	1.8439 [0.047]	−0.0221 [0.049]	0.9980	123	8.7
UV	Slope (b)/[S_b] [mAU/(μg/ml)]	Intercept (=peak height, a)/[S_a] [mAU]	Correlation coefficient	LOD[a] (ng/ml)	RSD (%)
Bromazepam	0.9631 [0.017]	0.0123 [0.015]	0.9987	86	8.1
Lorazepam	0.8081 [0.006]	0.0038 [0.005]	0.9997	36	3.4
Nitrazepam	1.1618 [0.012]	−0.0011 [0.011]	0.9995	51	4.8
Clonazepam	1.1956 [0.013]	0.0067 [0.012]	0.9995	52	5.1
Nordiazepam	1.7111 [0.020]	0.0025 [0.018]	0.9994	55	5.3
Flunitrazepam	1.2324 [0.009]	0.0008 [0.008]	0.9997	35	3.3
Midazolam	0.4511 [0.009]	0.0008 [0.008]	0.9982	99	9.3
Diazepam	1.2422 [0.014]	0.0137 [0.013]	0.9994	55	5.3

[a] LOD values were calculated from the regression line according to LOD=$a+3S_{y/x}$ [16,17]; a=calculated intercept and $S_{y/x}$=standard deviation of the calibration graph.

benzodiazepines showed a linear relationship between concentration and analytical signal (peak height) up to at least 2 mg/l for each compound. Both detection techniques exhibited excellent correlation, high sensitivity and precision. The relative standard deviation (RSDs) were in the range between 1.2 and 8.7% for electrochemical detection and somewhat higher (between 3.3 and 9.3%) for UV detection. The detection limits (LODs) listed in Table 2 were calculated from the regression lines according to LOD=y_B+3S_B [16,17]. The calculated intercept, a, was used as an estimate of y_B, the blank signal itself, and the standard deviation of the calibration line, $S_{y/x}$, was used as an estimate of the standard deviation of the blank, S_B. The values obtained in such a way are somewhat higher, but appeared to be more reliable and accurate estimates than those usually calculated from signal-to-noise ratios. Nonetheless the LODs that can be reached with both detection techniques are sufficiently low for benzodiazepine determination also in a biological matrix even at therapeutical levels [6,18]. Somewhat higher sensitivity and lower detection limits can be achieved by electrochemical detection, in cases where the compounds contain more than one electroactive (=reducible) substituent in the accessible potential range. This is due to the fact that in contrast to UV detection the detector signal in electrochemical detection depends on the number of electrons transferred during the electrochemical reaction. Thus, benzodiazepines, such as clonazepam, flunitrazepam and nitrazepam, containing an additional nitrophenyl substituent, yield a higher analytical signal, when a more negative potential (<−800 mV) is applied, than other, equally concentrated benzodiazepines without nitrophenyl substituent. As a consequence an increase in sensitivity and lower detection limits can be gained for those substances.

4. Conclusion

The optimized assay presented in this paper represents a highly versatile and rapid method for the determination of most common benzodiazepines by means of low cost and simple instrumentation. A

simple isocratic system together with serial UV–reductive electrochemical detection was developed and optimized, that can be used in high sensitivity and selectivity for qualitative and quantitative screening of benzodiazepines. Even compounds, such as bromazepam, lorazepam and midazolam, that are likely to cause problems in benzodiazepine analysis either due to short retention time, co-elution or easy decomposition could be determined successfully and selectively. Its high selectivity makes the assay equally suitable for pharmacokinetic studies, determination of benzodiazepines in dosage forms and in biological or forensic samples, though for this purpose a clean-up procedure had to be developed. Especially when complex forensic samples have to be analyzed, e.g., post-mortem blood samples, the combination of two detection modes proves to be very useful and very often unambiguous interpretation of the chromatograms obtained by UV detection is only possible with reference to the electrochemically detected chromatograms. A direct comparison of the performance of reductive electrochemical detection and UV detection is demonstrated in the chromatograms in Fig. 7. In Fig. 7a an extract obtained after liquid–liquid extraction with chloroform of a partly putrified post-mortem sample was analyzed as described above. A known amount of *n*-methylclonazepam (I.S.) had been been added to the sample as internal standard. It is obvious, that in this case only reductive electrochemical detection permits the unambiguous identification and quantification of diazepam and its main metabolite nordiazepam, whereas the UV technique is vitiated by irrelevant matrix components. The chromatograms obtained from extracts of blood samples of a drug addicted person and of a defendant in a road traffic accident are shown in Fig. 7b and c. In both cases unequivocal interpretation of the chromatograms is made easier due to the complementary information obtained from two different detection modes.

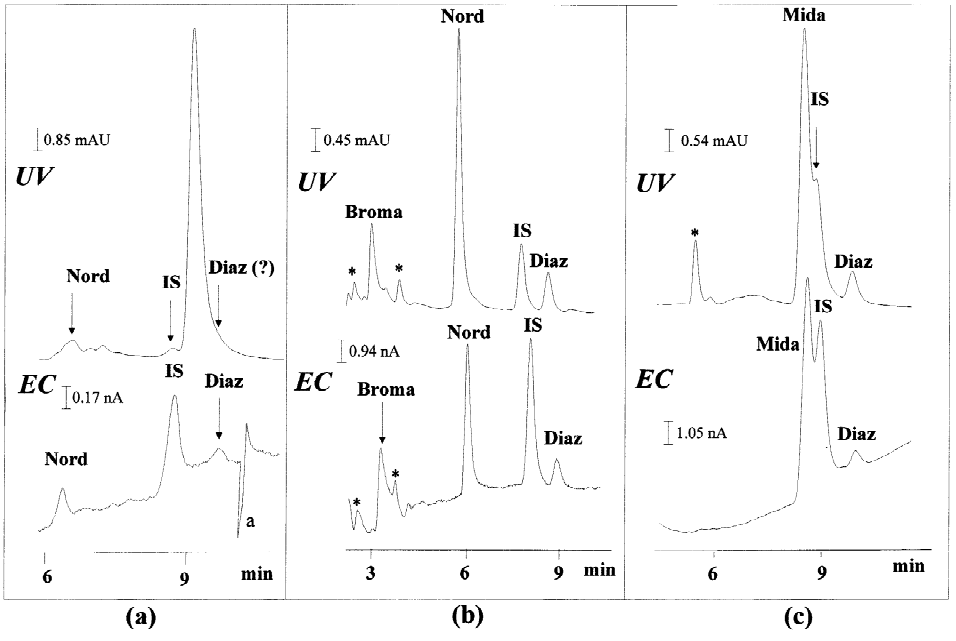


Fig. 7. Chromatograms of forensic casework samples obtained with UV detection at 250 nm and reductive electrochemical detection at −1400 mV (vs. Ag/AgCl). I.S.=Internal standard (*n*-methylclonazepam); *=unidentified matrix peaks. (a) Extract of post-mortem blood: Nord=nordiazepam (40 ng/ml); Diaz=diazepam (14 ng/ml); a=signal caused by incidental mercury drop dislodge. (b) Positive blood sample of a drug addicted person: Broma=bromazepam (433 ng/ml), Nord=nordiazepam (802 ng/ml), Diaz=diazepam (191 ng/ml). (c) Extract of blood of a defendant in a road traffic accident: Mida=midazolam (1665 ng/ml), I.S.=internal standard (*n*-methylclonazepam) (500 ng/ml), Diaz=diazepam (95 ng/ml).

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