Application Bulletin

Of interest to:	Organic chemistry; Pharmaceutical industry; Biochemistry, biology, medicine	B 3, 4, 8

Polarographic determination of diazepam in body fluids and pharmaceutical preparations

Summary

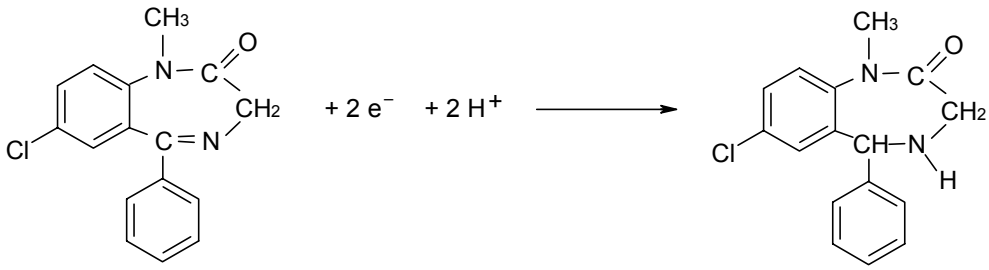
Diazepam belongs to the 1,4-benzodiazepine group of compounds, which are used for medical purposes as tranquillisers and antidepressants.

This bulletin describes the determination of diazepam in tablets and body fluids (blood, serum, urine) by means of differential pulse polarography. If a Britton-Robinson buffer pH = 2.8 with a methanol volume fraction of 20% is used as the supporting electrolyte then a distinct reduction peak is obtained at –0.73 V; this allows diazepam concentrations even below 0.05 µg/mL to be determined in blood. The necessary sample preparation steps are also dealt with in this bulletin.

Theory

Diazepam is very sparingly soluble in water, but easily soluble in methanol, chloroform, n-pentane, diethyl ether and diluted mineral acids. Sulphuric acid with a concentration of $c(H_2SO_4)$ = 0.1 mol/L hydrolyses the diazepam and thus prevents its exact determination. Using the supporting electrolyte described above ensures that diazepam is dissolved without the occurrence of unwanted hydrolysis.

At ca. –0.73 V the –CR=N– group in diazepam is reduced to –CHR–NH– by accepting two electrons (as well as two H^+ ions):



Instruments and accessories

- 746 VA Trace Analyzer with 747 VA Stand
  or
  757 VA Computrace
- Magnetic stirrer
- Centrifuge
- Analytical balance (minimum resolution 0.1 mg)
- Drying cabinet
- Desiccator
- Rotary evaporator
- Mortar, separating funnel, volumetric flasks, measuring cylinders, graduated and bulb pipettes, beakers

Reagents

Only reagents of the highest purity and ultrapure water are used for the preparation of the solutions. The organic solvents should have a degree of purity suitable for HPLC.

- n-Pentane
- Methanol
- Methanol : ultrapure water = 1 : 1, i.e. $\phi(CH_3OH) = 50\%$
- Sodium hydroxide c(NaOH) = 4 mol/L; this corresponds to a mass concentration of 160 g NaOH per litre ultrapure water
- Phosphoric acid $w(H_3PO_4) = 85\%$
- Supporting electrolyte: Britton-Robinson buffer pH = 2.8:

  200 mL methanol and 1.75 mL $w(H_3PO_4) = 85\%$ are added to a 1000 mL volumetric flask and made up to the mark with ultrapure water. 200 mL of this solution are placed in a beaker and then 160 mL methanol and 640 mL ultrapure water are added. The pH value of the solution is adjusted to 2.8 with c(NaOH) = 4 mol/L. The supporting electrolyte is stored in a tightly sealed glass bottle.
- Diazepam standard solutions:
  - Stock solution with a concentration of 1000 ppm:

    Ca. 0.5 g pure substance are dried for 24 h at ca. 70 °C in a drying cabinet and then allowed to cool down in a desiccator. 100.0 mg of the diazepam prepared in this way are weighed into a 100 mL volumetric flask, dissolved in 50 mL methanol, made up to the mark with ultrapure water and mixed. The stock solution is stored in the dark in a cool place; it is stable for about one week.
  - Working solutions with concentrations of 50 ppm, 100 ppm and 200 ppm:

    The diazepam working solutions are prepared from the 1000 ppm stock solution by diluting with $\phi(CH_3OH) = 50\%$. They must also be stored in the dark in a cool place and can be used for two to three days.

Sample preparation

1. Tablets

10 tablets are weighed to determine their average mass and then finely ground in a mortar. The amount of tablet powder corresponding to the average mass of an original tablet is then weighed into a beaker. 35 mL methanol are added, the beaker is covered with a watch glass and the solution is stirred for 20 min on a magnetic stirrer. When this «extraction time» has elapsed the mixture is rinsed quantitatively with $\phi(CH_3OH)$ = 50% into a 50 mL volumetric flask, made up to the mark and mixed very thoroughly. The sealed volumetric flask is placed in a cool dark place to allow precipitation of the insoluble residues.

2. Blood, serum, urine

15.0 mL blood are taken from the patient in the normal manner (addition of heparin to prevent blood coagulation) and immediately placed in a 60 mL separating funnel already containing 40 mL n-pentane. The blood sample is extracted for 2 min with intensive shaking, afterwards the two phases are allowed to separate (this separation time should be kept as short as possible). The blood phase is then transferred to a second separating funnel containing 20 mL n-pentane. The pentane phase is transferred to a centrifuge tube. The blood is extracted for a second and third time in the same way and the individual pentane extracts are combined in the centrifuge tube. The sample is then centrifuged for 10 min at 7500 min^{-1}. Afterwards the n-pentane is distilled off in a rotary evaporator at 70 °C. The resulting dry residue is dissolved in 500 µL methanol and then rinsed into the polarographic vessel with 14.5 mL supporting electrolyte. Serum and urine samples are prepared in the same way.

Analysis

1.00 mL tablet extract and 19.0 mL supporting electrolyte or the correspondingly prepared blood, serum or urine sample (see above) are placed in the polarographic vessel and purged with nitrogen for 5 min. (The washing bottle for the nitrogen attached to the VA Stand should also be filled with the supporting electrolyte.) The polarograms are then recorded under the following conditions:

Method / amplitude	DP / –50 mV
Electrode	DME or SMDE
U.start	–0.50 V
U.end	–0.95 V
Sweep rate	12.5 mV/s

The peak potential of diazepam lies at ca. –0.73 V.

The concentration is determined by two-fold standard addition.

Remarks

- A blank containing the chemicals used but no sample material has to be recorded (baseline) and taken into consideration when the results are calculated.
- The absolute mass of diazepam in the polarographic vessel including the standard additions should not exceed 200 µg as this represents the upper limit of the linear working range.
- The polarograms obtained are slightly asymmetric, which, however, has no influence on the reproducibility and accuracy of the results.
- Further tablet constituents that might also be electrochemically active do not interfere with the polarographic diazepam determination.
- Apart from diazepam other compounds of the 1,4-benzodiazepine group (e.g. nitrazepam) as well as 1,5-benzodiazepines can also be determined polarographically. In those cases the polarograms may show additional reduction peaks, particularly if the compounds contain nitro groups.
- In tablets marked as containing 2 mg diazepam per tablet 1.984 ± 0.021 mg diazepam were found. Two further samples yielded a diazepam content of 1.94 ± 0.034 mg.

  A blood sample from a patient showed a content of 0.960 µg diazepam/15 mL or 0.064 µg diazepam/mL ca. 2 h after the tablets had been taken.

Literature

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==================== METROHM 693 VA PROCESSOR (5.693.0020) ====================
 Method  Diazdet .mth          OPERATION SEQUENCE
 Title   Detn.of Diazepam in Pharmaceutical Products

      Instructions   t/s   Main parameters              Auxiliary parameters
 1    SMPL/M               V.fraction    1.000 mL      V.total       50.0 mL
 2    DOS/M                V.added      19.000 mL
 3    PURGE
 4    STIR           300.0 Rot.speed     2000 /min
 5    (ADD
 6      NOP          15.0
 7      SEGMENT            Segm.name    diaz
 8      ADD>M              Soln.name    dizstd        V.add         0.100 mL
 9    ADD)2
10    END

 Method: Diazdet                SEGMENT
                                  diaz

      Instructions   t/s   Main parameters              Auxiliary parameters
 1    (REP
 2      0PURGE
 3      0STIR
 4      0ME
 5      0PMODE             U.ampl         -50 mV       t.meas        20.0 ms
                           t.step        0.80  s       t.pulse       40.0 ms
 6      SWEEP        39.2  U.start        -500 mV      U.step          10 mV
                           U.end          -950 mV      Sweep rate    12.5 mV/s
 7      0MEAS
 8    REP)1
 9    PURGE
10    STIR               Rot.speed     2000 /min
11    END

 Method: Diazdet                DOCUMENTATION

 Auto form feed  no

 COPY  Reports, Curves                          TO Destination
 Curve Smth Sbst:diazepam VR:**                 Printer
 Report Full                                    Printer
 Curve Cal Sbst:diazepam                        Printer
 Report MethSpc                                 Printer
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Fig. 1: *Method for the polarographic determination of diazepam in tablets (performed on the 693 VA Trace Analyzer): Operation Sequence, Segment and Documentation.*

Method: Diazdet

SUBSTANCES
diazepam - diaz

Recognition

U.verify	-725 mV
U.tol (+/-)	30 mV
U.width min	10 mV
U.width max	200 mV
I.threshold	250 pA

Display / Plot

I.scale	auto
U.div	50 mV/cm
U.begin	mV
U.end	mV

Baseline

Type	linear
Scope	whole
dU.front	auto
S.front	auto
dU.rear	auto
S.rear	auto

Evaluation (for peaks only)

Quantity	I.peak

Calibration 94-02-01 15:10

Technique	std.add.
Curve type	linear

Coefficients

Y.reg	-4.922e-08
Slope	-2.439e-05
Nonlin.	
Mean dev.	2.712e-10

Additions

Soln.name	dizstd			
Mass conc.	200 mg/L	g/L	g/L	g/L
Range min	g/L	g/L	g/L	g/L
Range max	g/L	g/L	g/L	g/L
M.conc./cm	g/L	g/L	g/L	g/L

Method: Diazdet

CALCULATION
max. 15 lines

Quantity	Formula (R##, C##, A##)	Res.unit	Sig.dig.
diazepam	R1=MC:diazepam*158.6*50/(1000*50)	mg/50 ml	4

Fig. 2: *Method (continued): Substances and Calculation.*

```
==================== METROHM 693 VA PROCESSOR (5.693.0020) ====================
 Determ.      :  02011347              User:                      Date:  94-02-01
 Modified     :  94-02-01 14:01:41     Run :  1                   Time:  13:47:19
 Sample table:  -
--------------------------------------------------------------------------------
  Pos.  Ident.1/S1     Ident.2/S2     Ident.3/S3     Method.call    Sample size/S0
                              1.0                                        158.1 mg
--------------------------------------------------------------------------------
 Method   :  Diazdet
 Title    :  Detn.of Diazepam in Pharmaceutical Products
 Remark1  :  Ag/AgCl(3MKCl) reference electrode
 Remark2  :  Tab.wt. 30mL methanol.Dil. to 100.00mL with M-QW
--------------------------------------------------------------------------------

  Substance  :  diazepam                                          Comments
  Mass conc. :     39.56 mg/L              Mass      :  39.56 ug
  MC.dev.    :     0.416 mg/L (1.05%)      Add.mass  :     20 ug
  Cal.dev.   :     -                       V0.sample :      1 mL

              VR   U/mV    I/nA    I.mean  Std.dev.  I.delta   Comments
              --   ----    ----    ------  --------  -------   --------
              00   -724   -64.36   -64.69   0.4725
              01   -724   -65.03
              10   -724   -96.20   -96.44   0.3452   -31.75
              11   -724   -96.68
              20   -723   -128.8   -128.7   0.1539   -32.25
              21   -724   -128.6

 Substance    Techn.      Y.reg/offset    Slope        Nonlin.      Std.add.mass
 ---------    ------      ------------    -----        -------      ------------
 diazepam     std.add.      -6.456e-08   -3.264e-05                       20 ug

                                SOLUTIONS
                                 max. 40
================================================================================

 Soln.name    Pos.    Std.subst.    Mass conc.        Remark
 ---------    ----    ----------    ----------        ------
 dizstd         -     diazepam       200.0 mg/L


 C#  Workg.com.var  Remark
 --  -------------  ------

 Final results                          +/- Res.dev.    %      Comments
 -------------                          ------------    -      --------
   diazepam =     1.984 mg/50 ml            0.021     1.05
```

Fig. 3: *Full report for the determination of diazepam in tablets.*

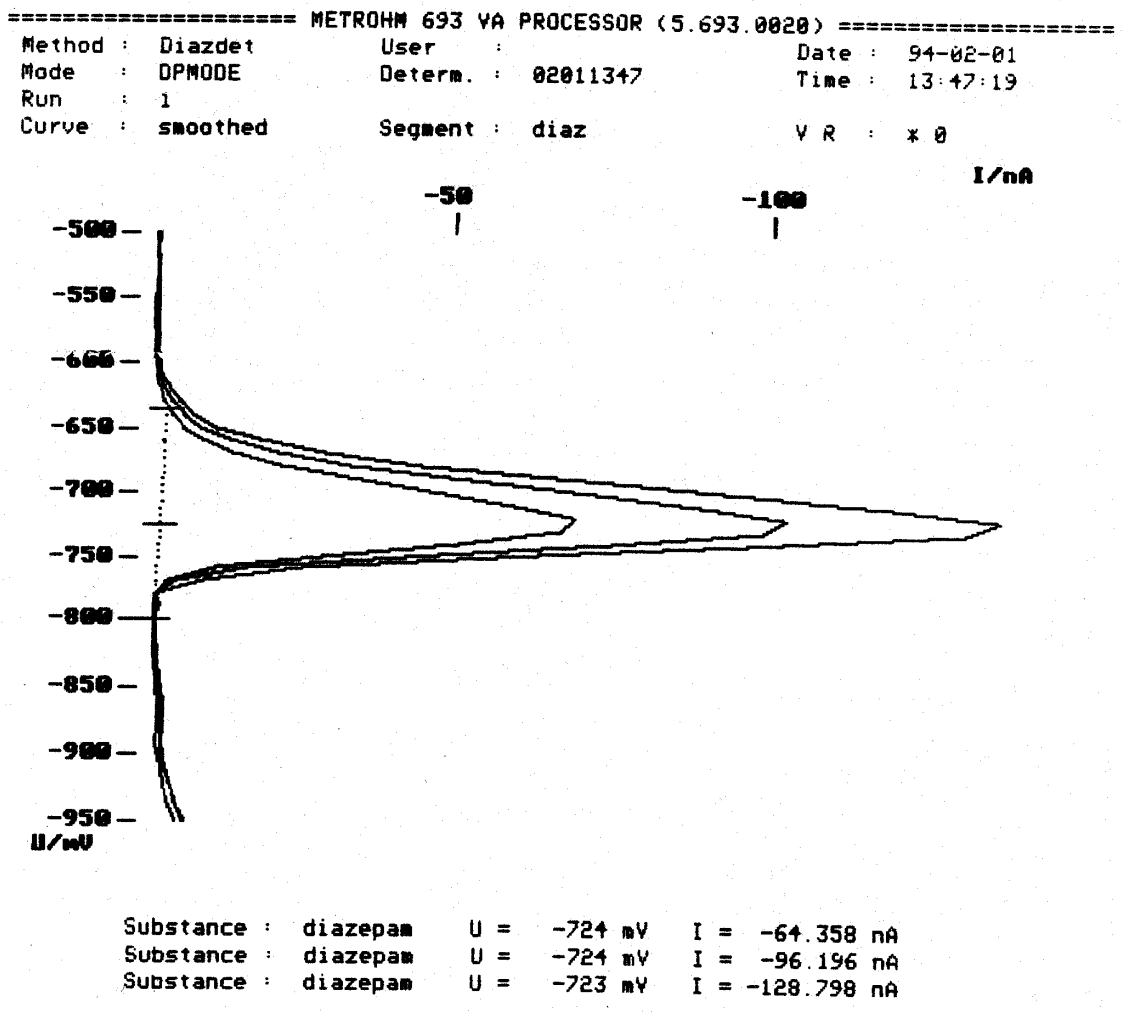

Fig. 4: *Polarograms for the determination of diazepam in tablets.*

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==================== METROHM 693 VA PROCESSOR (5.693.0020) ====================
 Determ.       :  05120947             User:                      Date:  94-05-12
 Modified      :  no                   Run :  1                   Time:  09:47:59
 Sample table:  -
-------------------------------------------------------------------------------
  Pos.  Ident.1/S1    Ident.2/S2    Ident.3/S3    Method.call    Sample size/S0
                                                                        15.0 mL
-------------------------------------------------------------------------------
 Method   :  Diazdet
 Title    :  Detn.of Diazepam in Blood Samples (Polarographic)
 Remark1  :  Ag/AgCl (3M KCl) reference electrode
 Remark2  :  15.0 mL B/R buffer 2.8 pH. This is for "blank"
-------------------------------------------------------------------------------

  Substance : diazepam                                          Comments
  Mass conc.:    14.14 ug/L          Mass      : 212.2 ng
  MC.dev.   :     2.35 ug/L (16.6%)  Add.mass  :     1 ug
  Cal.dev.  :     -                  V0.sample:     15 mL

              VR    U/mV    I/nA    I.mean   Std.dev.  I.delta    Comments

              00    -719   -0.5130  -0.5448   0.0450              front overlapping
              01    -715   -0.5766
              10    -724   -2.765   -2.818    0.0757   -2.274
              11    -724   -2.872
              20    -723   -5.223   -5.312    0.1263   -2.493
              21    -723   -5.401

 Substance    Techn.     Y.reg/offset    Slope        Nonlin.      Std.add.mass

 diazepam     std.add.    -5.072e-10    -3.588e-05                         1 ug

                                  SOLUTIONS
                                   max. 40
===============================================================================

  Soln.name    Pos.    Std.subst.    Mass conc.       Remark

  dizstd        -      diazepam         50.0 mg/L


 C#   Workg.com.var   Remark


 Final results                        +/- Res.dev.   %      Comments

   diazepam =    0.212 ug/15 mL          0.035     16.6
```

Fig. 5: *Full report for the determination of diazepam in blood.*

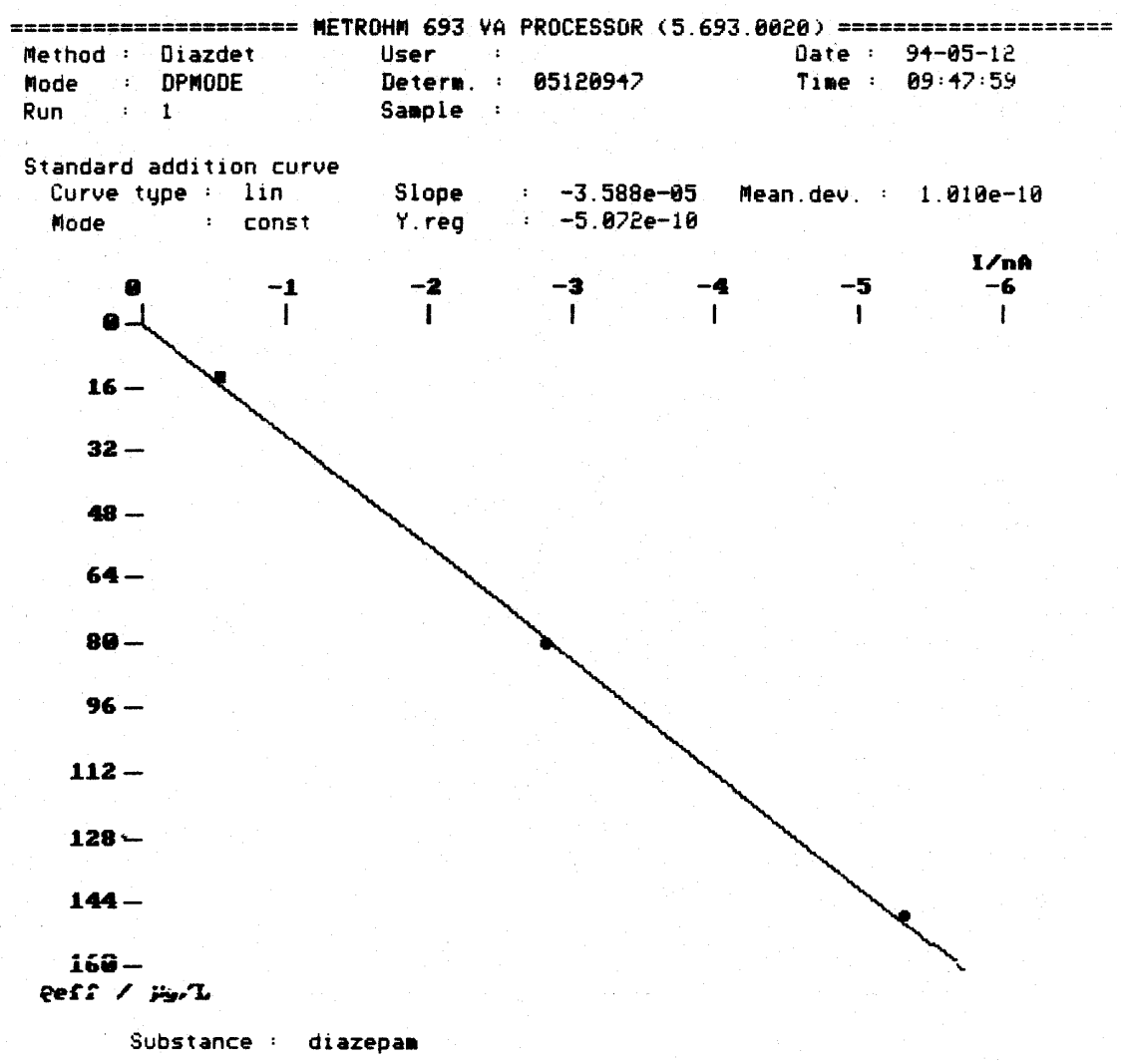

Fig. 6: *Standard addition curve for the determination of diazepam in blood.*