

Automated on-line solid-phase extraction–gas chromatography with nitrogen–phosphorus detection: determination of benzodiazepines in human plasma

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Abstract

An automated sample preparation module, the ASPEC (automated sample preparation with extraction columns) was interfaced with a capillary gas chromatograph (GC) by means of a loop-type interface. The system was optimized for the determination of four benzodiazepines in plasma. Extraction from the untreated plasma was carried out on disposable C_{18} cartridges and involved several washing steps. The analytes were desorbed with 2 ml of ethyl acetate and a 110- μ l aliquot of the eluate was injected into the gas chromatograph via the loop-type interface using fully concurrent solvent evaporation conditions. Detection of the benzodiazepines was carried out with a nitrogen–phosphorus detector (NPD). The ASPEC–GC–NPD was fully automated and could run unattended overnight. With a sample volume of 1 ml the procedure showed good linearity and repeatability in the range 5–50 ng/ml using a sample volume of 1 ml. The limits of detection in plasma were 0.5–2 ng/ml.

Keywords: Benzodiazepines; Medazepam; Diazepam; Clobazam; Midazolam

1. Introduction

Capillary gas chromatography (GC) is the separation method of choice for the trace analysis of complex mixtures because of its good separation efficiency and the availability of a wide range of sensitive and selective detectors. In the past decade important progress has been made in strategies that permit the on-line coupling of sample preparation and the GC analysis [1,2]. The liquid chromatographic (LC) part of such a system is generally used for analyte trace enrichment and sample clean-up utilizing solid-phase extraction (SPE). In such cases, SPE–GC is a better acronym than LC–GC. Whereas LC–GC and SPE–GC are already quite popular in

environmental analysis, their use is not widespread in the biomedical field. The determination of broxaterol and levopromol in plasma using on-line normal-phase LC–GC was reported by Ganesello et al. [3,4]. However, their procedures still require rather laborious sample pretreatment. Munari and Grob [5] discussed the determination of heroin in urine, but also in their case sample preparation could not be included into the total analytical procedure as a completely automated step.

Recently, we designed a system that enables the fully automated extraction and determination of analytes in plasma. That is, it includes extraction, clean-up, drying and transfer of the analytes to the GC part of the system. An ASPEC (automated sample preparation with extraction columns) system was interfaced to a GC equipped with a flame

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ionization detector (FID) via a trapping column [6]. The limit of detection for the antidepressive agent trazodone was 3 ng/ml for 1 ml of plasma. The above procedure was also applied to benzodiazepines, because most methods commonly used for their determination involve GC [7–9]. However, with the ASPEC-trapping column–GC–FID system the limits of detection were somewhat disappointing, viz. 30–50 ng/ml, due to interfering matrix constituents and unsatisfactory recoveries on the trapping column. Obviously, the system has to be redesigned and optimized for the determination of this type of analyte. In the present study, the selectivity was improved by using a nitrogen–phosphorus detector, because an electron capture detector which would also have been a good choice, could not be used in combination with large-volume transfers of ethyl acetate [6]. In order to improve analyte recovery, the trapping column was eliminated from the system and ethyl acetate was used for desorption instead of methanol. In the previous set-up, the trapping column was dried prior to its desorption. Unfortunately, in the present approach such a drying step could not be included. Consequently, the final eluate contained traces of water. Grob et al. [10] reported that water vapour had far less adverse effects on the deactivation of retention gaps and GC column materials than condensed water. The same authors also reported that large-volume injections of water containing 20% of butoxyethanol by means of a loop-type interface were successful [11]. Gerhart and Cortes [12] reported the successful introduction of 100- μ l water samples by a similar approach. This means that the injection of water-containing ethyl acetate at relatively high temperatures (i.e., water is injected as a vapour) should not necessarily have an adverse effect on the quality of the retention gap. Such relatively high temperatures (110–130°C) are commonly used for the loop-type transfer of ethyl acetate [13] using the fully concurrent solvent evaporation technique. The loop-type interface was selected as the transfer module for on-line ASPEC–GC because the majority of drugs which have to be analysed in plasma are not extremely volatile; it also has the advantage of easier optimization and operation [14].

In the present study, an SPE procedure for the extraction and clean-up of benzodiazepines in plasma

was optimized, automated using an ASPEC system and coupled on-line to GC–NPD. The latter aspect involved the large-volume injection of water-containing ethyl acetate solutions.

2. Experimental

2.1. Chemicals and reagents

Trazodone hydrochloride was purchased from Sigma (St. Louis, MO, USA). Clobazam, medazepam and midazolam were obtained from Solvay Duphar (Weesp, Netherlands). Diazepam was a gift from E.C. Goosens (Solvay Duphar). Nitrazepam was obtained from Bufa Chemie (Castricum, Netherlands). The structures of the benzodiazepines are shown in Fig. 1. Silica (particle size, 60–200 μ m) and aluminium oxide (particle size, 60–200 μ m) were obtained from Merck (Darmstadt, Germany). Sodium acetate trihydrate (Baker analysed HPLC-grade quality), hexane, methanol, ethyl acetate, acetonitrile and phosphoric acid were from J.T. Baker (Deventer, Netherlands). All solvents were glass distilled prior to use. Water was purified using laboratory-built distillation and purification equipment. Stock solutions (1 mg/ml) of each compound were prepared in methanol. Working standards were prepared daily by dilution with ethyl acetate. Plasma samples were spiked with an appro-

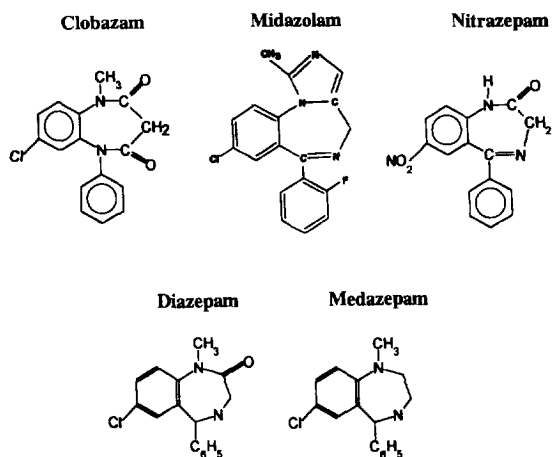


Fig. 1. Structures of benzodiazepines.

appropriate amount of the stock solutions. All solutions were stored in the dark at 4°C.

2.2. Instrumentation

The ASPEC system was from Gilson (Villiers-le-Bel, France). The Supelclean LC-18 (100 mg; 40 μm particles) disposable extraction cartridges (DEC) were purchased from Supelco (Bellefonte, CA, USA). The ASPEC was connected to a laboratory-built loop-type interface as shown in Fig. 2.

The loop-type interface consisted of two pneumatic Valco (Houston, TX, USA) six-port valves, a constant-flow controller (Pye Unicam, Cambridge, UK), a constant pressure controller (Pye Unicam) and a pressure meter (Pye Unicam). The six-port valves were controlled by a Kipp 5140 solvent programmer (Delft, Netherlands) for automated injection. The Kipp programmer was started by a remote ASPEC signal.

A Carlo Erba Instrumentazione (Milan, Italy) Model 5300 HRGC equipped with a NPD-40 thermionic detector (Carlo Erba) was used. The valves of the loop-type interface were connected to a 3 $\text{m} \times 0.32 \text{ mm}$ I.D. retention gap deactivated with diphenyltetramethylsilazane (DPTMDS) (B. Schilling, Zürich, Switzerland), a 2 $\text{m} \times 0.32 \text{ mm}$ I.D.

retaining precolumn and a 15 $\text{m} \times 0.32 \text{ mm}$ I.D. capillary GC column, both containing SPB-5 (Supelco) with a film thickness of 0.25 μm . Helium was the carrier gas at a column head pressure of 45 kPa. The pressure controller of the loop-type interface was set to 200 kPa. Connections were made with conventional glass press-frits, a glass press-frit Y-piece (BGB Analytik, Zürich, Switzerland) and a Valco T-piece. The Valco T-piece was connected to a 0.25 $\text{m} \times 2 \text{ mm}$ O.D. piece of silicone tubing which was led through a 24 V pinch solenoid valve (Type S 104; Sirai, Milan, Italy). This valve was controlled by a 24 V triggering signal on the GC and served as an early solvent vapour exit (SVE). The valve squeezed or opened the silicone tubing with a magnetic stamp. Ethyl acetate which remained in the Valco T-piece and the connecting capillary after injection was removed by a small helium purge flow through a 1 $\text{m} \times 50 \mu\text{m}$ I.D. fused-silica restriction coupled to the Swagelock T-piece.

2.3. Procedure for on-line ASPEC–GC–NPD

Each disposable extraction cartridge (DEC) in the ASPEC module was conditioned with 2 ml of methanol, followed by 1 ml of HPLC-grade water and 1 ml of 0.1 mol/l sodium acetate in HPLC-grade

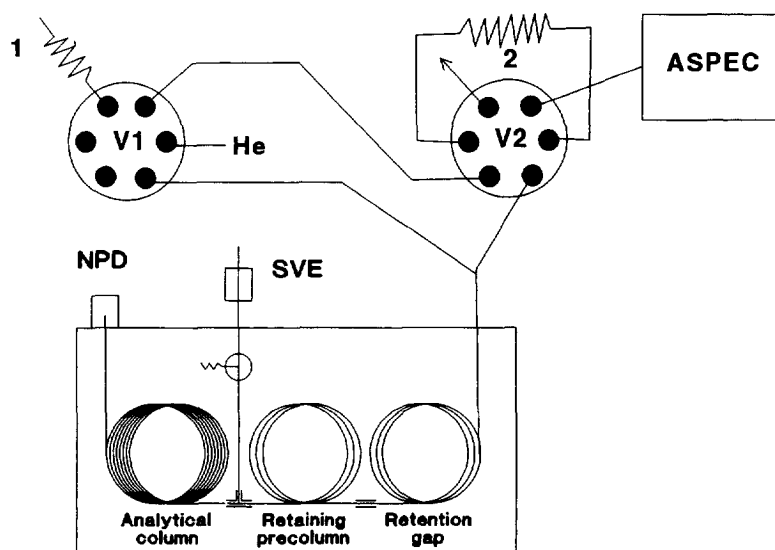


Fig. 2. System set-up for on-line ASPEC–GC–NPD. 1=purge leak restriction, 2=sample loop, V1, V2=six-port HPLC-valves. SVE=solvent vapour exit. For details, see text.

water. Next, 1 ml of plasma sample was loaded on the DEC. The cartridge was then washed with 2 ml of HPLC-grade water and 1 ml of water–methanol (9:1, v/v) in order to remove salts and polar matrix constituents. The cartridge was purged with 10 ml of air in order to remove most of the water. Finally, the analytes were eluted from the DEC with 2 ml of ethyl acetate and collected in a vial of the ASPEC.

With the ASPEC dilutor, 500 μ l of the final eluate were drawn from the collection vial and used to preflush and load the 110- μ l sample loop of the interface. Both 6-port valves of the interface were switched simultaneously to inject the contents of the sample loop into the retention gap of the GC. The injection time was 45 s, the SVE open time was 60 s. The injection temperature was 110°C (4-min hold time) with a subsequent linear increase to 300°C at 20°C/min. The final hold time was 5 min. After injection the sample loop of the interface was flushed with 2 ml of ethyl acetate.

3. Results and discussion

3.1. Set-up for large-volume injections of benzodiazepines

Using a loop-type interface constructed in-house, the introduction temperature was optimized for ethyl acetate. Flooding was observed up to 107°C which led to the selection of 110°C as the optimum injection temperature. Under these conditions, the most volatile analyte of the test mixture, medazepam, was quantitatively recovered when the response of large-volume injections was compared with 1- μ l

standard injections. This means that the loop-type interface can be used for the large-volume transfer of benzodiazepines, as was also found when coupling dialysis on-line to GC [15]. The performance of large-volume injection–GC–NPD was tested with a mixture of benzodiazepines using 0.11- and 1.00-ml samples. Repeatability and linearity data are shown in Table 1. For the 0.11-ml loop, the repeatability was found to be fully satisfactory at both the 10 ng/ml and 100 ng/ml levels, except for the rather polar nitrazepam at the lower level (R.S.D., 14%; $n=7$). This was probably caused by poor peak area integration due to peak tailing. The linearity was excellent in the 1–100 ng/ml range. For the 1-ml loop all analytical data were found to be fully satisfactory. The detection limits for the benzodiazepines using the detector in the NP mode were 20–30 pg (S/N ratio=3). In principle this corresponds with a detection limit (in concentration units) of 20–30 pg/ml when a 1-ml sample is used. However, when such a large volume was injected on the GC–NPD system several small peaks were detected which originated from the ethyl acetate and could only partially be removed by careful (re)distillation. Because of this “chemical noise”, the real detection limits for 1-ml injections were in the 50–150 pg/ml range.

3.2. Injection of “wet” ethyl acetate

Because the present ASPEC system is not equipped with a drying option, the final ethyl acetate eluate will contain some water. Therefore water will be injected into the GC system which has been stated to be permitted when a loop-type interface is used

Table 1
Repeatability and linearity of large-volume injection–GC–NPD

Compound	0.11-ml loop			1.00-ml loop	
	R.S.D. ($n=7$) (%)		r^2 ^a	R.S.D. ($n=7$) (%)	
	10 (ng/ml)	100 (ng/ml)		10 (ng/ml)	0.5–100 (ng/ml)
Medazepam	8	2	0.9997	2	0.9988
Diazepam	3	1	0.9999	1	0.9979
Clobazam	5	2	0.9999	1	0.9995
Nitrazepam	14	2	0.9997	2	0.9998
Midazolam	1	2	0.9997	2	0.9991

^a r^2 , Squared regression coefficient; 7 data points in duplicate.

which is operated at temperatures of 110–130°C when water is injected as a vapour (cf. above). In order to test the real potential of large-volume injections of such “wet” solutions, an ethyl acetate solution of benzodiazepines was saturated with water and 110- μ l portions were injected into a GC–FID system. The injections were performed at temperatures from 140°C down to 100°C (10°C steps). When injections were carried out at 110–140°C no deterioration of the chromatographic performance was observed even after 50 injections. At a temperature of 100°C, however, the FID response showed a tremendous increase in noise and a considerable “hump” eluting prior to the test analytes was present. At this temperature, the peak shapes deteriorated after a few injections of water-saturated ethyl acetate, which is most likely due to destruction of the deactivation layer. These results demonstrate the possibility of injection water-containing solutions at temperatures of 110°C and higher. However, it has to be emphasized that this approach self-evidently is limited to the determination of analytes which start to be eluted in the 200–230°C range.

3.3. Optimization of SPE of benzodiazepines from plasma

The SPE procedure used before for the extraction of trazodone and benzodiazepines was initially also applied here [6]. The C_{18} cartridge was conditioned with methanol, HPLC-water and 0.1 mol/l sodium acetate. Next 1 ml of plasma was loaded. Clean-up was performed by washing with 1 ml of a 0.01 mol/l

phosphoric acid solution and 1 ml of HPLC-grade water. The analytes were desorbed with 1 ml of methanol from which 110 μ l were injected into the GC system. However, using this procedure no benzodiazepines could be detected even at the 100 ng/ml level due to interfering matrix peaks and rapid retention gap degradation.

In order to be able to inject the C_{18} eluate directly into the GC, several other solvents and mixtures of solvents were tested (see Table 2). The main criteria were absence of interfering peaks on GC–NPD and the chromatographic performance after large-volume injections. For all elution solvents and mixtures tested, except for pure methanol, the desorption volume had to be increased from 1 to 2 ml in order to ensure maximum recovery. Fig. 3 shows the chromatograms of the analysis of 1 ml spiked plasma (100 ng/ml) eluted with ethyl acetate (trace A), hexane (trace B) and an ethyl acetate–cyclohexane azeotrope (trace C). The difference in selectivity is quite obvious. The apolar hexane only elutes the more apolar compounds; the peak which elutes at 16.3 min is a contaminant originating from the hexane, which also appeared in the azeotrope of ethyl acetate–cyclohexane. Trazodone was added in order to test the selectivity of the ASPEC–GC–NPD procedure for compounds eluting at relatively higher temperatures; nitrazepam was omitted from the mixture because of its rather poor peak shape at low ng/ml levels.

With the ethyl acetate–cyclohexane mixture satisfactory recoveries were obtained for the benzodiazepines and the matrix interferences were substantially

Table 2
Comparison of clean-up and analyte recovery for SPE of benzodiazepines from plasma on C_{18} modified silica using different elution and washing solvents

Method		Results		
Wash	Elution	Clean-up ^a	Recovery ^a	General remarks
Water	Methanol	--	+	Matrix interferences
Water	Ethyl acetate	-	+	Matrix interferences
Water	Ethyl acetate–hexane (10:30, v/v)	-/+	-/+	Poor repeatability
Water	Ethyl acetate–cyclohexane (azeotrope)	-/+	-/+	Retention gap lifetime limited
Water–methanol (95:5, v/v)	Ethyl acetate	-	+	Matrix interferences
Water–methanol (90:10, v/v)	Ethyl acetate	++	-/+	Matrix interferences
Water–methanol (90:10, v/v)	Ethyl acetate–hexane (10:30, v/v)	++	-	Poor repeatability
Water–methanol (80:20, v/v)	Ethyl acetate	++	--	Severe analyte loss

^a -- = very poor; - = poor, -/+ = fair, + = satisfactory; ++ = very good.

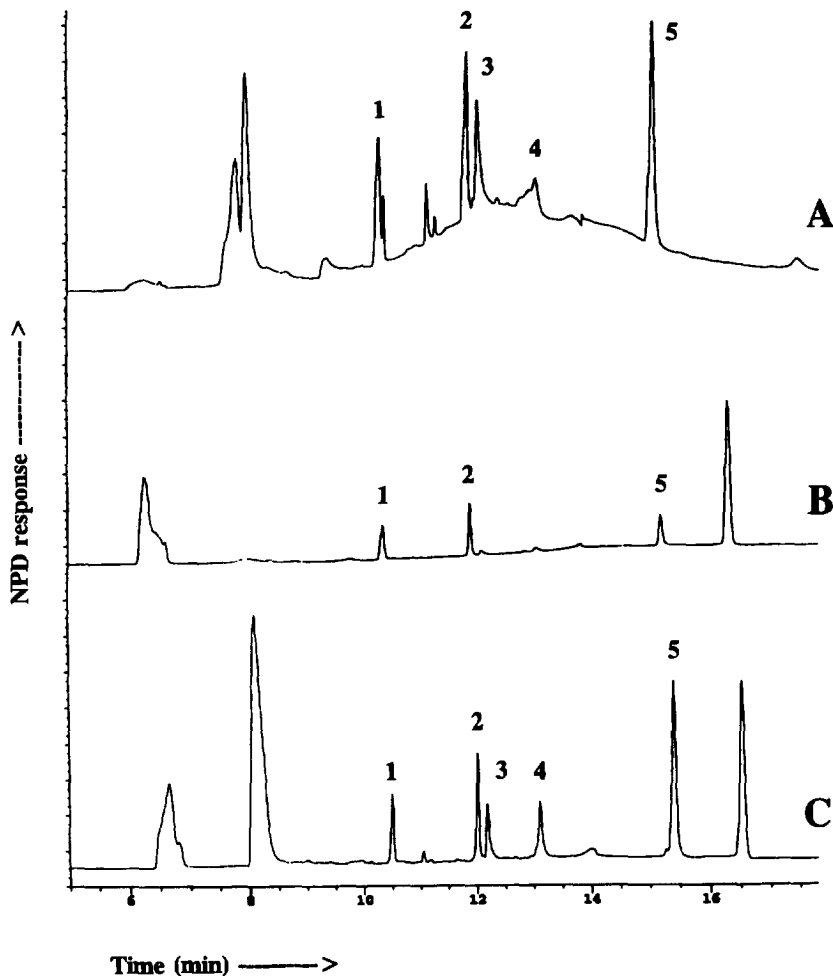


Fig. 3. SPE-GC-NPD chromatograms of 1 ml plasma spiked with 100 ng/ml of each of four benzodiazepines and trazodone and desorbed from C_{18} cartridges with 2 ml of (A) ethyl acetate, (B) hexane and (C) the azeotropic mixture of cyclohexane and ethyl acetate. Peaks: 1=medazepam; 2=diazepam; 3=clobazam; 4=midazolam; 5=trazodone. GC retaining precolumn, 3 m \times 0.32 mm I.D. BP-5 (film thickness 0.45 μ m). For details, see text.

reduced compared to the results with ethyl acetate. However, the interference was still too high in order to detect the test analytes at the low ng/ml levels actually required and 1.0-ml injections of the eluate caused deterioration of the retention gap after some 15 runs. The recoveries could be improved by omitting washing of the C_{18} extraction column with phosphoric acid which increases its polarity (recoveries, 65–80%). However, as was to be expected, the amount of matrix components also increased which decreased retention gap lifetime. Washing with 1 ml of water-methanol (90:10, v/v) was rather

successful, the larger part of the matrix interference now being removed. Here, ethyl acetate was the best elution solvent. Increasing the percentage of methanol to 20 vol.% caused a steep decrease in analyte recoveries, whereas a lower percentage (5 vol.%) was not sufficient to remove matrix material. The best compromise between clean-up and analyte recovery resulted in a final SPE procedure which now consists of conditioning with methanol, HPLC water and 0.1 mol/l sodium acetate. Next, 1 ml of sample was loaded. Clean-up was performed by washing with water-methanol (90:10, v/v) and

Table 3
Analyte recoveries and standard deviations (S.D.) from 1 ml water and plasma samples^a

Compound	Recovery (mean \pm S.D.) (%)	
	HPLC water	Plasma
Medazepam	70 \pm 6	65 \pm 6
Diazepam	60 \pm 6	55 \pm 9
Clobazam	50 \pm 14	50 \pm 11
Midazolam	65 \pm 7	65 \pm 10

^a Spiking level, 50 ng/ml; $n=5$.

HPLC-grade water. The analytes were eluted with 2 ml of ethyl acetate. Table 3 shows that the analyte recoveries and precision data did not differ significantly for spiked HPLC-grade water and plasma samples; or, in other words, the matrix has no adverse effects. Fig. 4 shows an ASPEC–GC–NPD chromatogram for 1 ml plasma spiked at the 50 ng/ml level. It clearly demonstrates the selectivity and sensitivity of the final procedure. In order to prevent carry-over, the sample loop of the interface

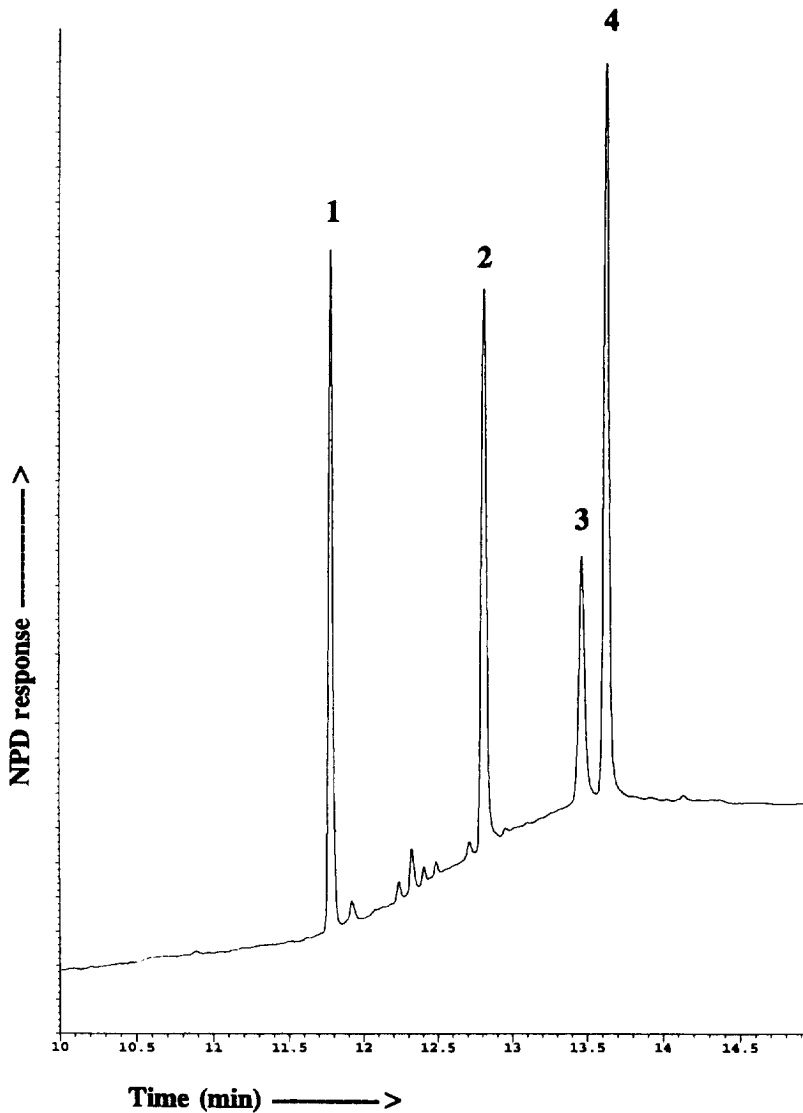


Fig. 4. SPE–GC–NPD chromatogram of 1 ml plasma spiked at the 50 ng/ml level with four benzodiazepines. Peaks: 1=medazepam; 2=diazepam; 3= clobazam; 4=midazolam. GC Retaining precolumn, 3 m \times 0.32 mm I.D. SPB-5 (film thickness 0.25 μ m). For details, see text.

had to be flushed with 2 ml of ethyl acetate after each analysis.

In an attempt to improve both clean-up and recovery, the more polar silica and aluminium oxide extraction materials were tested as an alternative to C_{18} . Unfortunately, with both materials the clean-up and recovery results were unsatisfactory.

3.4. Analytical data

The final ASPEC–GC–NPD procedure was tested for repeatability and linearity. Table 4 shows the results. The repeatability at the 50 ng/ml level ranged from 10 to 23% ($n=5$). However, with medazepam as an internal standard, the R.S.D. values improved to 2–7%. The linearity data were satisfactory for three out of four analytes (r^2 , 0.992–0.997). The somewhat unsatisfactory result for the polar clobazam (r^2 , 0.989) was not unexpected in view of its rather high R.S.D. value (23%) and low recovery (50%). The combined analytical data can be considered satisfactory for these low levels in plasma samples. The system was tested for robustness by analysing 50 plasma samples overnight and during the weekends. No decrease in system performance was observed which demonstrates the efficiency of the clean-up step. The sample preparation was carried out simultaneously with the GC analysis and the total analysis time required was 20 min.

The detection limits ranged from 0.5 to 2 ng/ml, which is better than reported for off-line procedures which use GC–NPD or GC–ECD (5–20 ng/ml) procedures [7–9]. Only for midazolam the same detection limit (0.5 ng/ml; 2 ml plasma sample) was

Table 4
Repeatability and linearity of ASPEC–GC–NPD of spiked 1 ml plasma samples

Compound	R.S.D. ^a (%)	r^2 ^b	LOD ^c (ng/ml)
Medazepam	10 (–) ^d	0.992	1
Diazepam	14 (3)	0.995	1
Clobazam	23 (7)	0.989	2
Midazolam	14 (2)	0.997	0.5

^a Relative standard deviation; $n=5$; spiking level, 50 ng/ml.

^b Range, 5–50 ng/ml; 6 data points.

^c Limit of detection; $S/N=3$.

^d R.S.D. with medazepam as internal standard.

reported after a rather laborious liquid–liquid extraction method and analysis of 10% (2 μ l) of the final extract using GC with surface ionization detection [16]. Actually, the limits of detection can be as low as 0.1–0.4 ng/ml by injecting a 10-fold larger aliquot (1.0 ml), i.e. essentially half of the total final eluate.

4. Conclusions

With an ASPEC module coupled on-line to GC–NPD via a loop-type interface, full automation of the set-up has been achieved. Large-volume injections of water-containing ethyl acetate were possible at an injection temperature of 110°C or above. Washing with water–methanol was essential to achieve satisfactory clean-up and enable re-use of the retention gap for at least 50 runs. Both the system sensitivity and selectivity were good and detection limits of 0.5 ng/ml were obtained for several benzodiazepines in 1 ml of plasma. The analytical data in the lower ng/ml range were fully satisfactory.

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