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Automated on-line dialysis for sample preparation for gas chromatography: determination of benzodiazepines in human plasma [1]

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Abstract

An on-line dialysis–solid-phase extraction–gas chromatographic (GC) approach has been developed for the determination of drugs in plasma, using some benzodiazepines as model compounds. Clean-up is based on performing the dialysis of 100 μl samples for 7 min using water as acceptor phase and trapping the diffused analytes on a PLRP-S copolymer precolumn. After drying of the precolumn with nitrogen for 15 min, the analytes are desorbed with ethyl acetate (275 μl) and injected on-line into the GC system via a loop-type interface. The system provides a very efficient clean-up, and offers the possibility of adding chemical agents which can help to reduce drug–protein binding and, thus, increase sensitivity. To demonstrate the potential of the described approach, the determination of benzodiazepines in plasma at their therapeutical levels is used as an example with flame ionization, thermionic and mass-selective detection.

Keywords: Automated on-line dialysis; Benzodiazepines; Gas chromatography; Plasma

1. Introduction

Capillary gas chromatography (GC) is the separation method of choice for the trace analysis of complex mixtures because a good separation efficiency can be obtained and several selective detectors are available. However, in many cases sample preparation still involves laborious method such as off-line liquid–liquid partitioning or, in other words, most GC applications are based on the off-line collection of extracts obtained by means of column liquid chromatography (LC) or solid-phase extraction (SPE), with subsequent preconcentration and re-injection of the separate fractions of interest into the GC part of the system.

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Important progress has been made through the development of efficient sample preparation strategies that permit on-line coupling to the final GC step. In this respect, coupled LC–GC or, rather, SPE–GC is highly attractive for a number of reasons: SPE provides very efficient clean-up of samples, direct transfer of the SPE eluate prevents losses of (volatile) compounds, and the whole procedure can be easily automated. Moreover, the use of uncoated capillary columns several metres long as so-called retention gaps allows the injection of large volumes of sample solutions into a GC column. This ensures complete transfer of the LC fraction of interest, and thus provides maximum analyte detectability [1,2].

As indicated above, on-line techniques such as SPE which are conventionally used for sample clean-up in LC can, in principle, be coupled to GC. A number of examples based on sample preparation by means of SPE have been reported, but most of these involve environmental analysis [2,3], and applications to biological samples still involve laborious sample treatment [4–6]. Recently, an automated procedure for the determination of drugs in plasma based on interfacing an ASPEC (automated sample preparation with extraction columns) unit to a GC system via a trapping column was reported [7]. In this instance, clean-up was carried out with disposable C_{18} cartridges, using various washing steps. The analytes were subsequently desorbed with methanol and the extract was diluted on-line and trapped on a PLRP-S copolymer precolumn. The precolumn was dried with nitrogen and finally the analytes were desorbed with ethyl acetate (120 μl) and injected on-line into the GC system via a retention gap using partially concurrent evaporation conditions.

In the last few years, dialysis has gained popularity as a sample preparation technique in the determination of traces of analytes in protein-containing matrices, because the use of a semi-permeable membrane offers the possibility of removing macromolecular sample constituents. In addition, if a trace-enrichment precolumn is incorporated in the set-up to overcome the dilution of the sample caused by the dialysis step, efficient sample clean-up and analyte enrichment can be combined in the system in a fully automated way. On-line dialysis has been successfully applied to a variety of biomedical, food and environmental sample types prior to LC analysis [8]. However, to our knowledge, the combination of dialysis and GC has been reported only twice [9,10]. In the former example, amines were determined in urine down to the 1 $\mu g\ l^{-1}$ level, but the injection volumes were only about 3 μl [9]. In the other study, five opiates present in plasma and blood were trapped on a polymer sorbent after dialysis, desorbed with acetonitrile and collected in vials using a fraction collector; further steps were carried out manually [10].

The aim of the present study was to demonstrate the potential of dialysis as a preparation technique for the on-line determination of drugs in plasma by SPE–GC. Several benzodiazepines (medazepam, diazepam, midazolam, *N*-desmethylclobazam and nitrazepam) were selected as model compounds because most methods commonly used for their identification and determination employ GC [11–13], and also because optimal conditions for dialysis of benzodiazepines have been established previously [14]. Various set-ups were studied for both water and plasma samples and a final procedure for the determination of these compounds in plasma is reported.

2. Experimental

2.1. Chemicals and reagents

Nitrazepam and diazepam were obtained from Bufa Chemie (Castricum, Netherlands), medazepam and midazolam from Solvay-Duphar (Weesp, Netherlands), *N*-desmethylclobazam from Hoffmann-LaRoche (Basle, Switzerland) and ethyl acetate (HPLC grade) from Riedel-de Haën (Seelze, Germany). All other chemicals and reagents were purchased from J.T. Baker (Deventer, Netherlands). Stock standard solutions (500 $\mu g\ ml^{-1}$) of each compound were prepared in methanol. Aqueous working standard solutions were daily prepared from the stock standard solutions by dilution with water. Plasma samples were spiked with an appropriate amount of the stock standard solutions. All solutions were stored at 4°C.

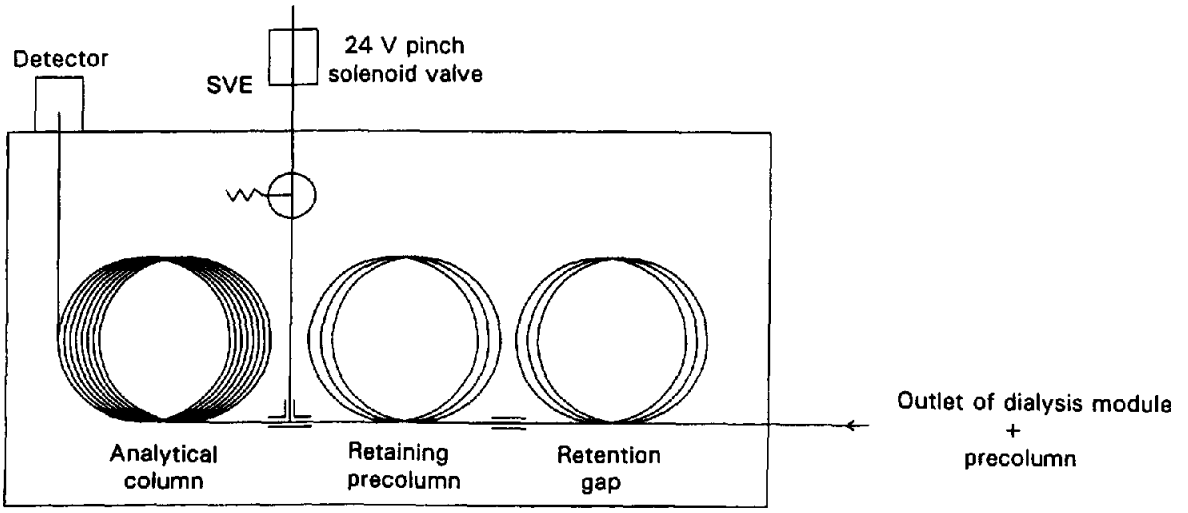

Fig. 1. Schematic representation of the GC system. For explanation, see text.

2.2. Dialysis and on-line trace enrichment

A Gilson (Villiers-le-Bel, France) ASTED system was used for dialysis of the samples. The system consisted of a Model 231 autosampling injector equipped with two 1 ml Model 401 dilutors in a slave configuration, a Model 99/50 rack for 128 vials of 860 μl and five reagent vials of 25 ml, and a six-port switching valve. The dialysis cell was made of poly(methyl methacrylate), with donor and acceptor channel volumes of 100 and 170 μl, respectively. A Gilson Cuprophane membrane with a molecular mass cut-off of 15 kDa was used. On the basis of previous results [14], water was selected as the donor phase for dialysis. Since no significant differences in analyte recovery were observed, water instead of a pH 7 buffer was also used as the acceptor phase (flow rate 3.0 ml min^{-1}) to avoid the injection of additional ions in the GC system. Before each run, the donor and acceptor channels were flushed with 2 ml of water; the precolumn was also conditioned with 2 ml of water. Next, 100 μl of sample were placed in the donor channel and dialysis was performed for 7 min at ambient temperature. After dialysis, the donor channel was cleaned with 2.0 ml of water–methanol (80:20, v/v), and the acceptor channel was flushed with 1.0 ml of acceptor phase.

For reconcentration, a 10 mm × 2 mm i.d. stainless-steel precolumn (acting as trapping column) slurry packed with PLRP-S, 100 Å, 15–25 μm (Polymer Laboratories, Church Stretton, UK) was used. After sample loading, the precolumn was dried with a nitrogen purge (pressure 3 bar; flow rate 120 ml min^{-1}). After the drying step, the analytes were desorbed with ethyl acetate, which was delivered by a Gilson Model 305 piston pump or a 10 ml Gilson Model 401 dilutor. Drying and desorption were automated by means of a multiport stream switch Must system (Spark Holland, Emmen, Netherlands), which consisted of two six-port valves and a timer.

2.3. Gas chromatography

Gas chromatography was performed on a Dualchrom 3000 (Carlo Erba, Milan, Italy) on-line LC–GC system equipped with a flame ionization detector (FID) or a thermionic detector (NPD). Samples were injected via a loop-type interface equipped with a 500 μl loop. The GC system (Fig. 1) consisted of a 3 m × 0.32 mm i.d. retention gap deactivated with DPTMDS (BGB Analytik, Zürich, Switzerland), a 3 m × 0.32 mm i.d. retaining precolumn and a 15 m × 0.32 mm i.d. analytical GC column, both of which contained SE-54 with a 0.25 μm film thickness (Alltech Associates,

Deerfield, IL). Connections were made with conventional glass press-fits and glass press-fit Y-pieces (BGB Analytik). The Y-piece was connected via a 15 cm × 0.32 mm i.d. fused-silica capillary to a Type 104 S 24 V pinch solenoid valve (Sirai, Milan, Italy), which served as a solvent vapour exit (SVE). In order to prevent dead volumes in the GC system, this valve was also connected to a 1 m × 50 μm i.d. fused-silica capillary, which served as a purge restrictor. The carrier gas was helium at an inlet pressure of 48 kPa. The SVE was automatically opened at the beginning of the GC run. After evaporation of the solvent, the injection valve was automatically switched (the system detects the end of the solvent evaporation by monitoring the decrease in the pressure with a delay time of 15 s) and the SVE was closed after a delay time of 45 s. The injection temperature was 110°C. After an initial hold time of 8 min, the temperature was increased to 300°C at 10°C min^{-1}, with a final hold time of 10 min.

Assays using mass spectrometric (MS) detection were performed on an HP 5890 Series II (Hewlett-Packard, Palo Alto, CA) chromatograph equipped with an HP 5971A (Hewlett-Packard) mass selective detector. A 100 μl aliquot of the samples was injected into a 5 m × 0.32 mm i.d. retention gap deactivated with DPTMDS (BGB Analytik), which was connected to a 2 m × 0.25 mm i.d. retaining precolumn and a 26 m × 0.25 mm i.d. analytical column, both coated with HP5-MS (film thickness 0.25 μm) (Hewlett-Packard). Helium at an inlet pressure of 60 kPa was used as the carrier gas. The injection temperature was 60°C, with a hold time of 4 min. Next, the temperature was increased to 280°C at 10°C min^{-1}, with a final hold time of 5 min.

2.4. Off-line dialysis–GC

Off-line experiments were performed by collecting the analytes (desorbed from the PLRP-S column with 1 ml of ethyl acetate pumped at a flow rate of 0.5 ml min^{-1}) in 2 ml glass vials (Bester, Amstelveen, Netherlands). Next, a 500 μl aliquot of the extract was injected into the LC–GC system.

2.5. On-line dialysis–GC

A schematic representation of the on-line system is shown in Fig. 2A. In order to prevent the injection of water into the GC system, valve V1 was switched after performing dialysis, and the precolumn was dried with a nitrogen stream for 15 min at ambient temperature, water being pushed to waste via valve V3. Next, valves V2 and V3 and the ethyl acetate pump were automatically activated, and the trapped analytes were transferred to the injection loop by pumping ethyl acetate at a flow rate of 0.5 ml min^{-1}. In all experiments the injector loop was partly filled, i.e. with 375 μl of eluent. After injection, valve V4 was switched and the trapping column and injection loop were cleaned with 2.0 ml of ethyl acetate.

Dialysis efficiencies were calculated by determining the recoveries in aqueous and in spiked plasma samples containing 1 μg ml^{-1} of each of the analytes. The recoveries were calculated by comparing the peak heights in the dialysed samples and the peak heights obtained after the direct injection of standard solutions in ethyl acetate containing an equivalent amount of drug. All experiments were performed in duplicate.

3. Results and discussion

3.1. Preliminary experiments

Two different approaches were initially tested to avoid the presence of water in the fraction of eluent from the trapping column injected into the GC system, in order to prevent deterioration of the retention gap and, thus, loss of chromatographic performance. Schematic diagrams of the set-ups used are shown in Fig. 2. In the first configuration (Fig. 2A), water was eliminated by drying the trapping column with a nitrogen stream for a given period of time, and the analytes were subsequently desorbed and transferred to the GC system by pumping ethyl acetate. As an alternative, analytes were eluted from the trapping column immediately after dialysis; the first traction eluted, which contained water, was sent to

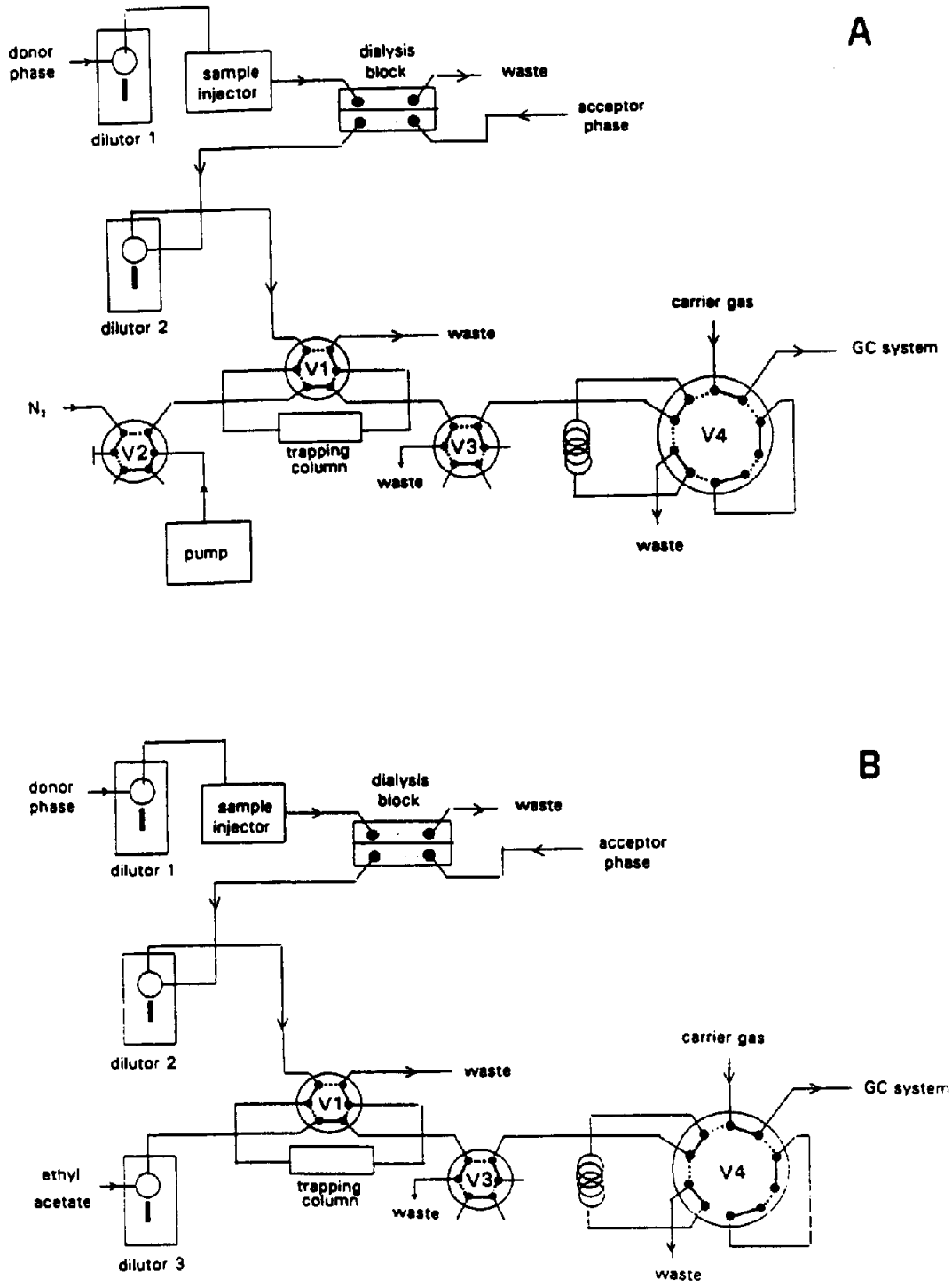

Fig. 2. Configurations tested for coupling dialysis to GC: (A) set-up used when water is eliminated by drying the trapping column and (B) set-up used when water is eliminated by discarding the first fraction of eluent.

Table 1
Conditions for on-line dialysis–SPE–GC of benzodiazepines in plasma

Unit	Process	Conditions
Dialyser	Conditioning	Donor channel: 2 ml of HPLC-grade water Acceptor channel: 2 ml of HPLC-grade water
	Sample loading	100 μl of plasma
	Dialysis	Time: 7 min Acceptor phase: HPLC-grade water at flow rate of 3.0 ml min^{-1}
	Cleaning	Donor channel: 2 ml of water–methanol (80:20, v/v) Acceptor channel: 1 ml of HPLC-grade water
Trapping column	Conditioning	2 ml of HPLC-grade water
	Drying	15 min of nitrogen purging
	Elution	375 μl of ethyl acetate injected on-line into the GC column
	Cleaning	2 ml of ethyl acetate

waste and the analyte-containing fraction was subsequently sent to the GC system (Fig. 2B).

Preliminary off-line experiments with water samples showed that if no drying step is incorporated in the procedure, at least the first 50 μl fraction eluting from the trapping column must be discarded to prevent water from entering the retention gap. These studies also showed that the benzodiazepines were completely eluted from the precolumn with about 200 μl of ethyl acetate. This indicates that the margin between loss of performance caused by transfer of water and loss of the analytes due to discarding too large a volume will be small. It was indeed observed that when using a conventional LC pump, the analyte recoveries had a poor precision. Therefore, in the on-line configuration in Fig. 2B, a 10 ml dilutor was used to desorb the analytes at a flow rate of 0.36 ml min^{-1}. However, even when only 50 μl of eluent were discarded, the analyte recoveries were not really satisfactory, viz. 30–65%.

When a drying step was carried out (Fig. 2A) and the total eluted fraction was transferred to the GC system, the recoveries of the analytes of interest typically were higher than 80%. This convincingly indicates that neither dialysis nor trapping, drying and desorption cause appreciable losses. In other words, with configuration B, a considerable percentage of the benzodiazepines is lost in the discarded 50 μl fraction of eluent. On the basis of these results, configuration A was selected as the best approach for on-line dialysis–SPE–GC. With this approach, no differences in analyte recoveries were observed for drying periods between 10 and 30 min. In order to be on the safe side and, thus, prolong the lifetime of the retention gap, a drying time of 15 min was selected. The conditions finally selected for the analysis of plasma samples are summarized in Table 1.

3.2. Analysis of plasma samples

Table 2 shows typical data on the recovery of benzodiazepines from spiked aqueous solutions and plasma using the dialysis–SPE–GC approach. The procedure is much less efficient when plasma instead of an aqueous solution is processed. This can be explained by the well known strong binding of these drugs to plasma proteins

Table 2
Recoveries of benzodiazepines from water and plasma samples using dialysis–SPE–GC [a]

Compound	Recovery (%) [b]	
	In water	In plasma
Medazepam	89 ± 9	6.5 ± 0.8
Diazepam	92 ± 4	16 ± 2
Midazolam	87 ± 9	5.7 ± 0.9
N-Desmethylclobazam	102 ± 6	14 ± 3
Nitrazepam	64 ± 6	41.0 ± 1.5

[a] Concentration of benzodiazepines in samples, 1 μg ml^{-1}. For experimental conditions, see text.
[b] Mean ± SD ($n = 3$).

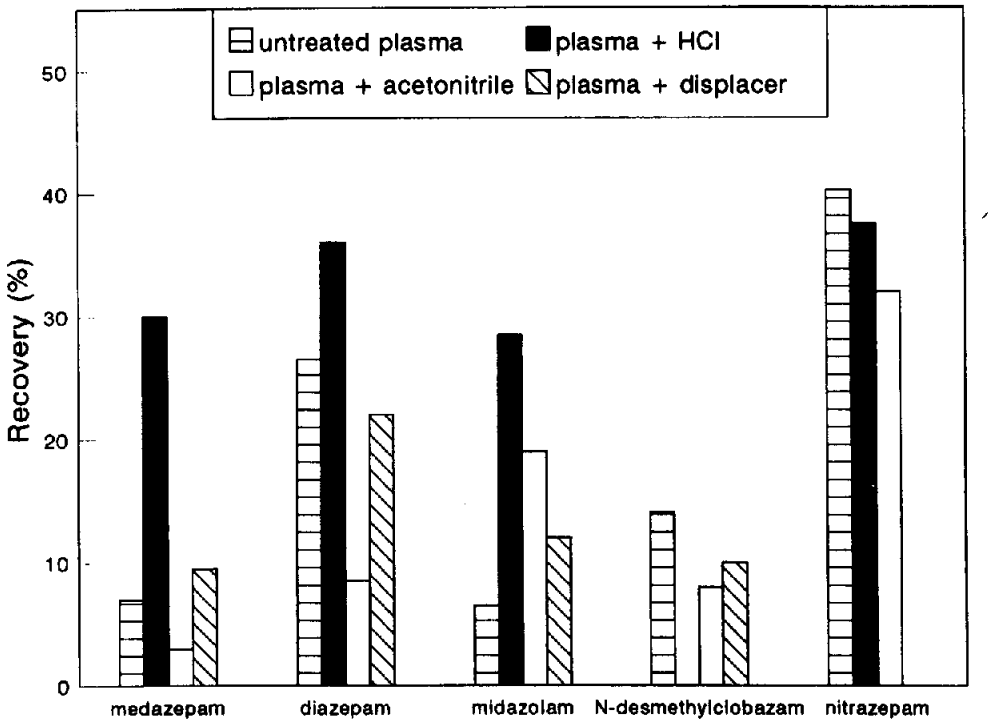

Fig. 3. Recovery of benzodiazepines (1 μg ml^{-1}) in untreated plasma, plasma at pH 4, plasma containing 20% (v/v) acetonitrile, and plasma containing 200 μg ml^{-1} of displacer. *N*-Desmethylclobazam was used to displace medazepam, diazepam and midazolam; nitrazepam was used to displace *N*-desmethylclobazam. For experimental details, see text.

[15]. Still, the chromatograms obtained for plasma samples showed that the system provides excellent clean-up of the samples. The selectivity was satisfactory even when FID was used (data not shown). However, because of the low free concentration of drug present in plasma samples, FID is not suitable for determining benzodiazepines in plasma at relevant (therapeutic level) concentrations. Self-evidently, analyte detectability can be improved considerably by using NPD instead of FID. Probably this exchange of detector will be satisfactory for most applications concerning these compounds ([16]; see also below). However, as a continuation of earlier work [17], we also studied different approaches to increase the free concentration of benzodiazepines before application of dialysis: (i) modification of the structure of the protein induced by a change in the sample pH or (ii) addition of an organic solvent and (iii) displacement of the analyte by a compound which binds to the same binding site on the protein.

Fig. 3 shows that the recoveries of medazepam, diazepam and midazolam substantially increased when 50 μl of 1 M HCl were added to 500 μl of plasma, which resulted in a pH value of about 4. No significant differences in the recoveries of the benzodiazepines from aqueous samples at pH 4 and 7 were observed, except for *N*-desmethylclobazam, which is not stable at pH 4; consequently, this approach was not tested for plasma samples containing *N*-desmethylclobazam. A further decrease in the sample pH did not provide a significant improvement, probably because of adverse effects due to interaction of the protonated analytes (positively charged at low pH) with negatively charged groups of the membrane [14]. On the other hand, the addition of 20% acetonitrile to the samples was not satisfactory (Fig. 3), probably because the presence of the organic solvent produces breakthrough of the analytes on the trapping column [17]. Reducing the dialysis time (to 3 min) and, thus, the total volume of acceptor phase pumped through the trapping column did not improve the results.

Table 3
Analytical data for on-line dialysis–SPE–GC–NPD of five benzodiazepines in plasma

Analyte	Concentration range (μg ml^{-1})	Pretreatment	Linearity [a]		Limit of detection (ng ml^{-1})	Within-day precision [b] ($n=6$)	Recovery ± SD (%)
			$y=a+bx$	R			
Medazepam	0.125–1.5	Acidification [c]	$a=-1.17$ $b=78.0$	0.9994	10	6	30 ± 2
Diazepam	0.125–2.0	Acidification	$a=-1.55$ $b=67.0$	0.9997	10	12	35 ± 4
Midazolam	0.100–2.0	Acidification	$a=-7.3$ $b=117.3$	0.9991	5	12	28 ± 3
N-Desmethylclobazam	0.10–1.0	None	$a=1.5$ $b=62.6$	0.9990	25	16	14 ± 3
Nitrazepam	0.10–1.5	None	$a=0.08$ $b=78.7$	0.9990	10	4	41.0 ± 1.5

[a] Six data points in duplicate.
[b] Determined at half of highest concentration in tested range.
[c] 500 μl of plasma + 50 μl of 1 M HCl.

Finally, because benzodiazepines are known to be bound to the so-called benzodiazepine site or site II of human serum albumin (HSA) [18], we used *n*-octanoic and *n*-decanoic acid (which strongly bind to this site) to displace the analytes of interest [14,17]. However, the presence of these fatty acids caused rapid deterioration of the GC column. As an alternative, a benzodiazepine different from those to be analysed was used as a displacer. The concentration of displacer in the samples was 200 μg ml^{-1}, which means that the ratio of concentration of displacer to concentration of HSA was about 2. The displacer was selected taking into account that its retention time should be longer than that of the analyte (to prevent overlap of the analyte peak and the large peak due to the displacer), and that it should exhibit a high affinity for HSA (high protein-binding degree) to increase the free fraction of the displaced drug as much as possible [17]. The results obtained with *N*-desmethylclobazam and nitrazepam as displacers are included in Fig. 3. Although the analyte recoveries do increase in most instances, this approach will obviously not be the first choice with any of the tested analytes.

Summarizing the data in Fig. 3, one can conclude that the addition of hydrochloric acid is the best approach to increase the sensitivity for three out of the five test compounds. This is particularly useful in the determination of medazepam and midazolam, because only a small fraction (<7%) of these drugs is recovered from untreated samples. The recovery of *N*-desmethylclobazam cannot be improved in the same way because of its low pH stability and also because, among the tested compounds, only nitrazepam could be used as a displacer (to avoid peak overlap). However, since the affinity of nitrazepam for HSA is lower than that of *N*-desmethylclobazam (recovery data in Table 2) no improvement was observed at a concentration of the displacer of 200 μg ml^{-1}. At higher nitrazepam concentrations, peak overlap occurred. Finally, for nitrazepam itself, no additional treatment is required.

3.3. Determination of benzodiazepines in plasma by on-line dialysis–SPE–GC

The performance of the total analytical set-up in Fig. 2A was tested by determining benzodiazepines in plasma at their therapeutic levels. For the quantification of medazepam, diazepam and midazolam, the samples were acidified to pH 4 prior to analysis. Acidification was performed automatically by the ASTED system. Table 3 summarizes relevant analytical data of the procedure. In this part of the study, NPD was used to increase the selectivity and analyte detectability.

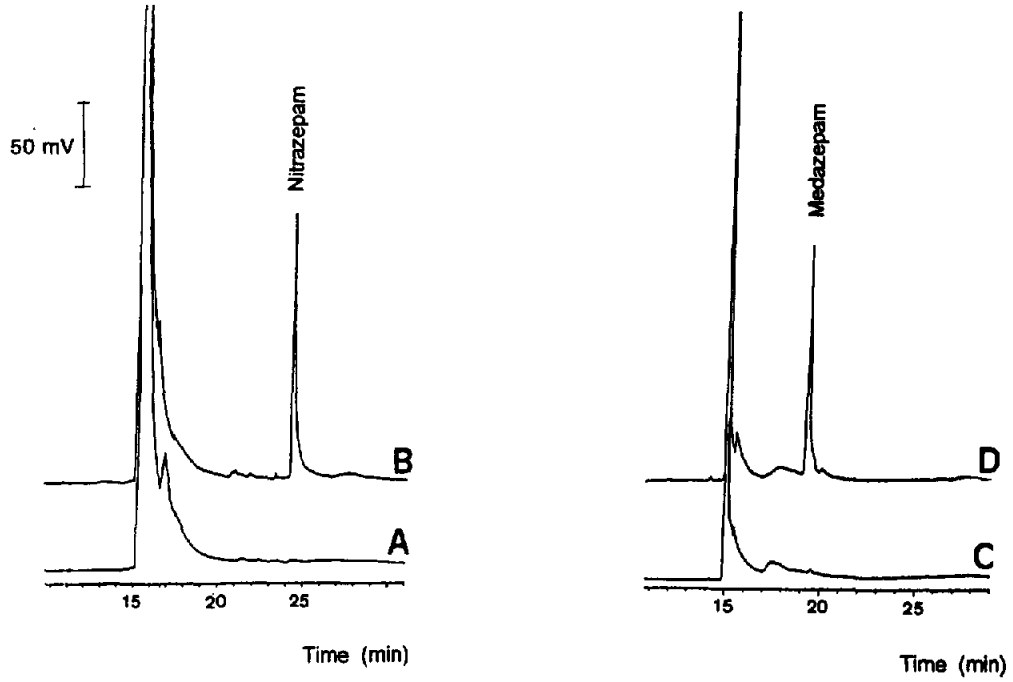

Fig. 4. On-line dialysis–SPE–GC–NPD of (A) untreated blank plasma, (B) untreated plasma spiked with 1 μg ml^{-1} of nitrazepam, (C) acidified blank plasma and (D) acidified plasma spiked with 1 μg ml^{-1} of medazepam. For experimental details, see text.

For a procedure involving (rapid) dialysis, the recoveries can be considered acceptable for all benzodiazepines, except *N*-desmethylclobazam. However, even for this analyte, quantification at therapeutic levels is obviously possible with satisfactory linearity and precision. With the present procedure, total analysis requires about 45 min. However, two samples can be simultaneously processed since clean-up and drying, etc., of a sample can be carried out during GC analysis of the previous sample. The dialysis block must be carefully cleaned after each run, otherwise serious deterioration of the GC performance occurs after a few runs. With the cleaning procedure used in this study (2 ml of water–methanol (80:20, v/v)), matrix components are completely removed from the membrane and tubing. As an example, typical chromatograms obtained for plasma spiked with nitrazepam (untreated) and medazepam (acidified plasma) are shown in Fig. 4.

Off-line dialysis experiments using GC with MS detection have confirmed the excellent selectivity of the dialysis–SPE–GC approach, as can be seen from Fig. 5, which shows chromatograms obtained for blank plasma and plasma spiked with medazepam.

4. Conclusions

The on-line coupling of dialysis, via a pre- or, rather, trapping column, to GC, i.e. dialysis–SPE–GC, appears to be an attractive technique for the determination of drugs and other compounds which do not require derivatization prior to GC analysis in plasma.

With the present set-up, clean-up of the samples is very efficient, and satisfactory selectivity is obtained with both FID and NPD. Further, after analyte reconcentration on the trapping column, desorption with ethyl acetate allows the on-line introduction of all analytes into the GC part of the system, which dramatically increases analyte detectability. If water is eliminated from the trapping column by rapid drying with nitrogen, at least 60 samples can be processed without replacement of the retention gap. Occasional cleaning of the GC precolumn (every 100 injections) is recom-

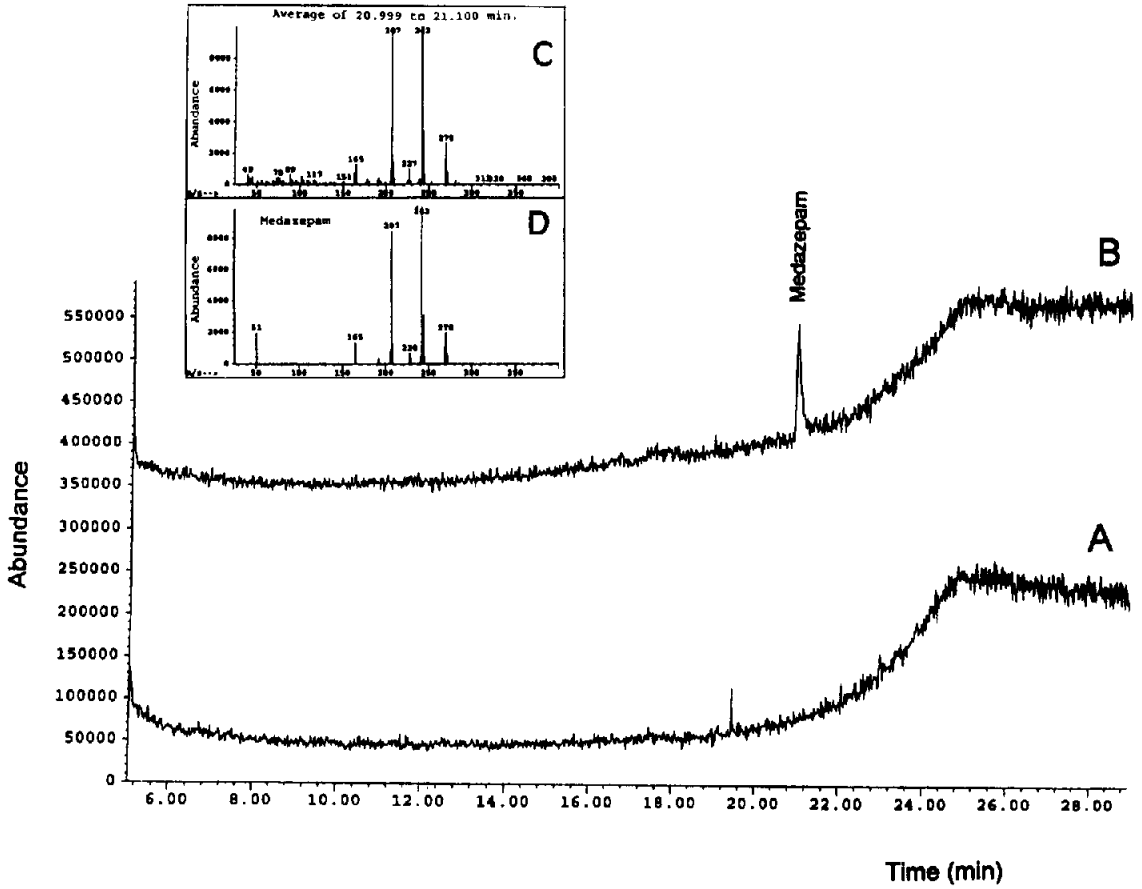

Fig. 5. Total ion current trace obtained by combining dialysis–SPE off-line with GC–MS for the analysis of acidified plasma (500 μl of plasma + 125 μl of 1 M HCl): (A) blank and (B) spiked with 1 μg ml^{-1} medazepam. Insets show (C) the mass spectrum obtained for medazepam in the spiked sample and (D) the mass spectrum obtained from the NBS library. Volume of sample injected, 1 μl.

mended to ensure adequate performance of the GC separation.

Finally, as regards the application selected for the present study, although the sensitivity is limited by the high degree of protein binding of the analytes, the detection limits obtained are comparable to those reported for other GC assays based on destructive sample pretreatment [11,19] because the on-line SPE–GC approach allows the injection of the total extracts. Moreover, the analytical system shows good compatibility with several chemical agents often used to increase the free concentration of protein-bound analytes. In the present instance, simple acidification of the samples helps to increase the recovery of some strongly bound benzodiazepines by factors of 2–5. The analysis, including the addition of HCl, can be fully automated.

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