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# Direct LC analysis of five benzodiazepines in human urine and plasma using an ADS restricted access extraction column

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**Abstract**

An alkyl-diol-silica (ADS) precolumn was used for the direct and on-line extraction of several benzodiazepines from serum and urine. The protein component of the biological sample was flushed through the ADS column, while simultaneously extracting the benzodiazepine compounds in the pores of the ADS stationary phase. The role of hydrophobic interactions in the extraction mechanism was confirmed. Column switching was employed to elute the extracted analytes from the ADS column into a high-performance liquid chromatography reverse-phase C18 column for the isocratic separation and UV detection of the benzodiazepines. Sample preconcentration via large volume injections was possible, improving the limits of detection. The calculated clonazepam, oxazepam, temazepam, nordazepam and diazepam detection limits were 38.8, 24.2, 31.7, 31.3, 45.0 ng/ml in serum, respectively, and 48.4, 24.5, 31.7, 33.1, 52.9 ng/ml for urine, respectively. The method was linear over the range of 50–10 000 ng/ml in both matrices with an average linear coefficient ($R^2$) value of 0.9918. The injection repeatability and intra-assay precision of the method were evaluated over ten injections, resulting in a percent relative standard deviation $< 5\%$. The ADS extraction column was robust, providing many direct injections of biological fluids for the extraction and subsequent determination of benzodiazepines. 

*Keywords:* Benzodiazepine analysis; ADS extraction precolumn; Direct injection; Reverse-phase chromatography; Serum; Urine

## 1. Introduction

Benzodiazepines represent a class of drug compounds administered for a wide range of clinical disorders. They have been prescribed as anti-anxiety agents, muscle relaxants, tranquilisers and anticonvulsants [1]. Benzodiazepines can lead to sudden death if misused [2], and have been the subject of abuse in suicide and criminal cases [3,4].

The extensive use and potential abuse of this class of compounds demands an accurate and rapid method for analysis. The established methodologies for benzodiazepines in serum and urine have been the subject of a recent review article [1]. The more common analysis methods

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include chromatographic techniques such as gas [5] and liquid chromatography [6], which have also been combined with mass spectrometry [7–9] for enhanced separation and detection capabilities. Alternative methodologies such as immunoassay [10,11] have been developed; however, the cross-reactivity of the antibodies often responds to a range of benzodiazepine compounds.

With the exception of some immunoassays, the analysis of benzodiazepines is preceded by sample preparation in the form of liquid–liquid extraction [12], solid-phase extraction [7,13,14] or solid-phase microextraction [15–17]. These sample preparation approaches can suffer from lengthy extraction times, excess use of solvents and poor automation capabilities. In addition, off-line extraction procedures increase the chance of sample loss or contamination.

One promising in-line sample preparation technique is the use of restricted access materials (RAM), which fractionate a sample into the protein matrix and the analyte fraction [18–20]. The RAM column allows the direct and multiple injections of untreated pertinacious fluids by preventing access of macromolecules to the bonded phase. Simultaneously with this size exclusion process, low molecular weight compounds are extracted and enriched, via partition, into the phase's interior. One recently developed example of a bio-compatible column for the direct drug analysis, alkyl-diol-silica (ADS), has been reported in the literature [21,22].

The objective of the present research was to develop a simple method for the ADS column extraction and rapid determination of several benzodiazepines in serum and urine samples. On-line extraction with coupled columns enabled direct injections, easy automation capability (less chance of sample loss or contamination), and decreased total analysis time, yielding a more reproducible and higher sample throughout. Previous determination of benzodiazepines by direct injection via column coupling [23,24] waas performed using different classes of precolumns and the extraction was not based on the same principle as the ADS restricted access material. Furthermore, the mechanism of extraction was not investigated in these studies, limiting the value for future method developments with these precolumns. Isocratic mobile phases were used to confirm the existence of hydrophobic interactions responsible for the extraction, elution and separation of the benzodiazepines compounds on the ADS and analytical columns, and this understanding can potentially simplify the time required for future ADS method development. To our knowledge, this is the first report of an ADS restricted access material applied to the direct injection and simultaneous extraction of several benzodiazepines in both serum and urine samples.

## 2. Experimental

### 2.1. Chemicals and materials

All solvents were HPLC grade or better and purchased from Caledon (Georgetown, Ont., Canada). The benzodiazepines (see Fig. 1) were purchased from Radian International (Austin, TX, USA) as 1 mg/ml methanol solutions and stored at 4 °C. Deionized water, from a Barnstead/Thermodyne NANO-pure ultrapure water system (Dubuque, IA, USA), was used for dilution of the standards.

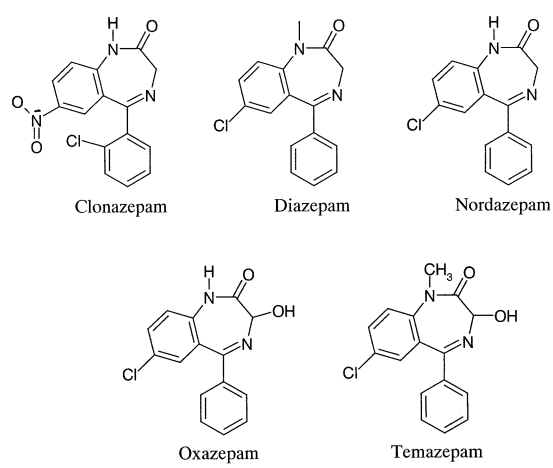


Fig. 1. Structures of the benzodiazepines evaluated in this study.

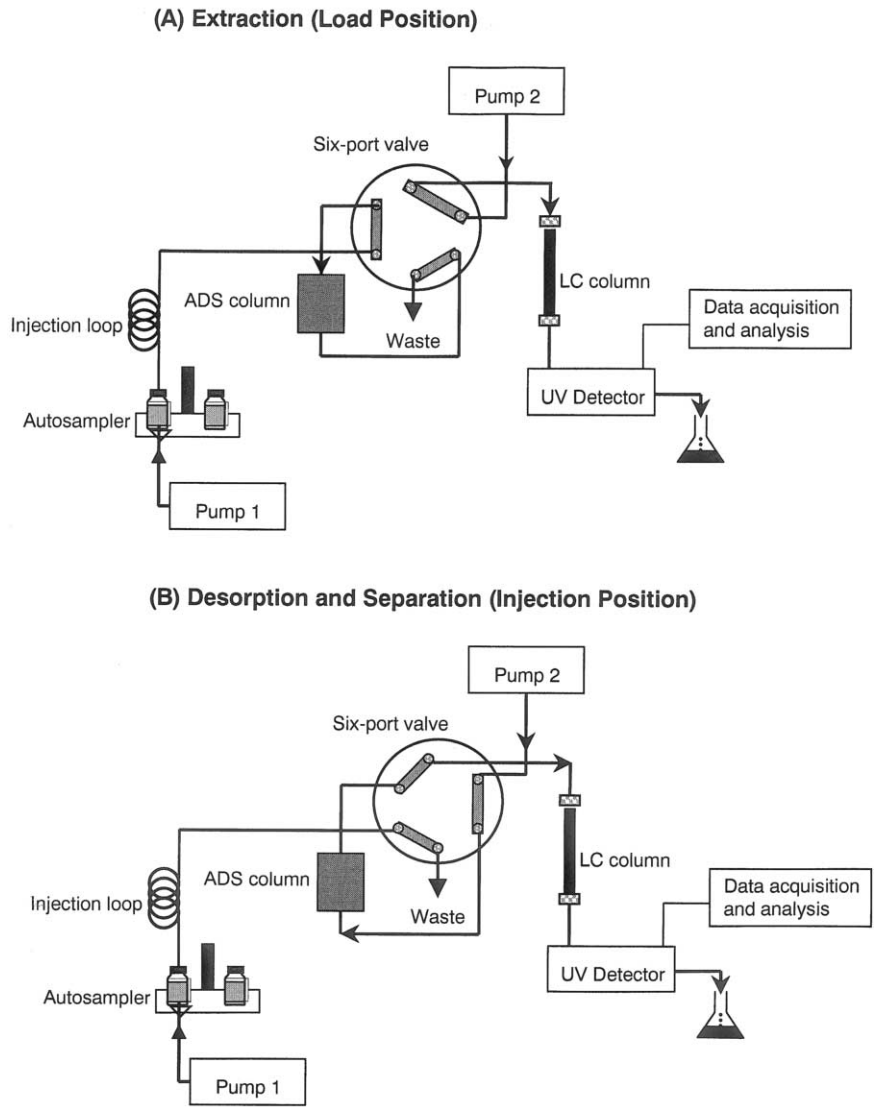


Fig. 2. Schematic representation of the ADS-HPLC configuration: (A) load position (extraction), (B) injection position (desorption).

### 2.2. Instrumentation and analytical conditions

The ADS-HPLC configuration is shown in Fig. 2. A Hewlett-Packard (Palo Alto, CA) HPLC system (model 1050) complete with autosampler and multiple wavelength UV detector was used as pump 1. The HP 1050 autosampler and pump were used to load a 100 μl aliquot of the benzodiazepine-spiked samples onto the ADS extraction column using a mobile phase of water–methanol (95:5 v/v) at a flow rate of 1.0 ml/min. After a predetermined wash time, pump 2, a TosoHaas HPLC pump model 6010 (Montgomeryville, PA, USA), was used for delivery of the elution and separation mobile phase of water–methanol (54:46 v/v). UV detection was performed at 230 nm. The LiChroCART® 25–4 mm filled with LiChrospher® RP-18 ADS, 25 μm Article Number 50187 extraction column was supplied by Merck KGaA (Darmstadt, Germany). The chro-

matographic column was a Supelcosil C18 column (5.0 cm × 4.6 mm i.d.; 5 μm particle size) from Supelco (Bellefonte, PA). A LiChrosorb® RP-18 guard column (1 cm × 4.6 mm) from Supelco (Bellefonte, PA) was installed at the inlet of the chromatographic column for protection of the analytical column. Elution of the extracted compounds from the ADS column and separation by the reverse-phase HPLC column was accomplished with pump 2 using a mobile phase of water–methanol (54:46 v/v) at a flow rate of 1.0 ml/min.

The determination of an equilibrium constant, for each benzodiazepine compound on the ADS column, was performed by removing the six-port valve and directly connecting the column to pump 1 and measuring the retention time of each eluting compound at 230 nm. The equilibrium constant values were used to aid in the optimization of the ADS-HPLC method development.

### 2.3. Preparation of urine and serum samples

Urine and serum samples were collected from a drug-free healthy volunteer. Any precipitated material was removed by centrifuging the sample at 10 000 × *g* for 10 min. The five benzodiazepines were directly spiked into the supernatant of the biological samples over a range of 0.05–10 μg/ml and directly injected by the autosampler at a draw speed of 50 μl/min.

## 3. Results and discussion

### 3.1. Extraction step

The ADS packing material possessed two different chemical surfaces. Hydrophilic electroneutral diol groups were bound to the external surface of the spherical particles, and this inert layer protected the sorbent from contamination by proteins, allowing direct and multiple injections of biological fluids. The inner surface of the porous particles contained a C18 alkyl hydrophobic bonded phase that was responsible for simultaneous extraction of the target compounds.

To establish a high recovery of the benzodiazepines from the serum sample, the extraction mobile phase must have the appropriate chemical composition to ensure high affinity of the benzodiazepines with the hydrophobic bonded phase, while remaining compatible with plasma proteins. Typically, the percentage of organic phase in the extraction mobile phase is limited to $< 15\%$ to prevent proteins from precipitating [25]. The elution profile of a blank serum sample from the ADS extraction column was recorded by connecting the column directly to the UV detector. As shown in Fig. 3, under mobile phase conditions of 95:5 water:methanol (v/v), the protein fraction of the serum sample produced a large response in signal. However, this component was eluted from

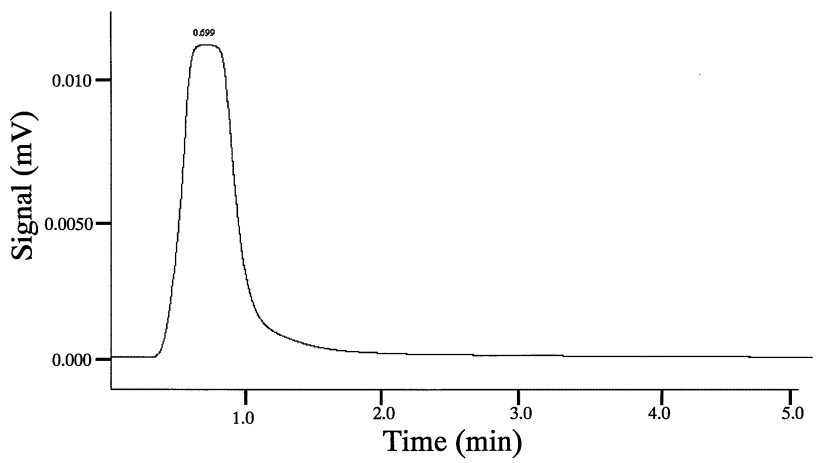


Fig. 3. Elution profile of the blank serum sample from the ADS extraction column. Chromatographic conditions: injection volume, 100 μl; mobile phase, water–methanol (95:5 v/v); flow rate, 1.0 ml/min; detection, $\lambda = 230$ nm.

the column over a period of 4 min as indicated by a return in the baseline value. The elution profile for a blank sample of urine produced a similar profile. Determination of this time value is critical, as it represents the point in the extraction process when the mobile phase may be switched for the elution and subsequent separation of the extracted analytes. Using the same experimental conditions, an elution profile for each benzodiazepine (1.0 μg/m in water) was recorded. In all instances, no elution of the benzodiazepine compounds was observed over a period of 60 min, indicating complete retention of the drug compounds on the ADS column. The development of a direct injection and on-line extraction method for benzodiazepine analysis in biological fluids, such as serum and urine, was now possible.

### 3.2. Desorption of the extracted benzodiazepines from the ADS column

The polarity of the mobile phase could be adjusted to ensure the rapid desorption of the compounds from the ADS fiber, while still allowing their separation on the analytical column. The polarity of the mobile phase was decreased with the addition of methanol and the elution profile of each benzodiazepine from the ADS column was re-evaluated. It is important to ensure the selected mobile phase will provide quantitative transfer of the extracted benzodiazepines from the ADS precolumn to the series connected analytical column. Using Eq. (1), and the capacity factor ($k$) of each evaluated benzodiazepine, the distribution constant ($K$) was calculated as follows:

$$K = k\frac{V_m}{V_s} \quad (1)$$

where $V_s$ is the volume of the stationary phase and $V_m$ the volume of the mobile phase. Table 1 summarizes the capacity factor, distribution constant and log $P$ value of each benzodiazepine compound. The actual or predicted value of log $P$ was obtained using the ACD/I-Lab Web service [26]. The log $P$ value of the drug compound was related to its distribution constant, confirming a hydrophobic or reverse phase type interaction between the drug and the C18 groups located in the pores of the ADS stationary phase material [27]. An increase in the benzodiazepine compound's log $P$ resulted in a longer elution time from the ADS column, and hence a higher distribution constant. This interaction was important to understand to ensure the compound could be extracted and desorbed under different mobile phase conditions at the appropriate time and, therefore, the log $P$ value was a convenient parameter for the optimization of the ADS-HPLC method development. For example, high extraction efficiencies of the hydrophobic benzodiazepines (high log $P$ values) were only possible when the sample injection was performed under polar mobile phase conditions (95:5 water:methanol (v/v)). In contrast, switching to a non-polar mobile phase was necessary to ensure complete elution of the extracted compounds to the analytical column, but this was only possible after the serum proteins had been completely removed from the ADS column.

Table 1
Calculated distribution constants for the selected benzodiazepines on an ADS precolumn

| Compound | Capacity factor ($k$) | Distribution constant ($K$) | Log $P$ |
|---|---|---|---|
| Clonazepam | 5.94 | 4.31 | 1.28 |
| Ozazepam | 6.37 | 4.62 | 2.17 |
| Temazepam | 7.29 | 5.28 | 2.20 |
| Nordazepam | 11.71 | 8.49 | 2.93 |
| Diazepam | 13.29 | 9.63 | 3.08 |

Experimental conditions: mobile phase, water–methanol (54:46 v/v); flow rate, 1.0 ml/min; volume injected, 20.0 μl; detection, $\lambda = 230$ nm.

In its most convenient form, the ADS-HPLC experimental set-up requires a mobile phase composition that provides complete elution of the extracted analytes from the ADS column, but still provides the necessary chromatographic separation of the compounds on the analytical column. Since the analytical column possessed a similar stationary phase chemistry (C18 reverse phase), the previously determined distribution constants were useful to predict the separation capability of the analytical column. If the distribution constant of each benzodiazepine is significantly different,

effective separation will be possible on the analytical column under the same elution conditions for the ADS precolumn.

To test this hypothesis, the analytical column was connected to the HPLC pump 1, autosampler and UV detector, and a 5 μg/ml standard mixture of benzodiazepines injected using various ratios of water:methanol (v/v) as the mobile phase. Fig. 4 shows that the elution behaviour of the benzodiazepine compounds, under the optimized ADS desorption conditions, was similar on both columns. Excellent separation of all five compounds on the analytical column was therefore possible (resolution value $\geq 1.5$) with an isocratic mobile phase of 54:46 water:methanol (v/v). This mobile phase composition represented the lowest possible polarity, important for desorption of the extracted compounds from the ADS column, while still maintaining the necessary chromatographic separation, and this optimized composition was used for all remaining experiments.

### 3.3. Serum and urine analysis

The heterogeneity of biological samples complicates benzodiazepine analysis as the direct injection of the sample into a chromatographic system is prohibited by the presence of many contaminates and interferents. However, the time-consuming requirement of sample preparation can be greatly eliminated with an ADS extraction precolumn that fractionates the protein component from the hydrophobic analytes. Employing the optimized extraction, column switching time and analyte elution, a simple, isocratic ADS-HPLC method was developed for direct injection and analysis of benzodiazepines in biological samples. Both serum and urine samples were spiked over a range of concentrations (0.05–10 μg/ml) with the five benzodiazepine compounds, and a 100 μl aliquot was injected directly into the ADS-HPLC system. Under mobile phase conditions of 95:5 water:methanol (v/v), the benzodiazepines were extracted and preconcentrated by the ADS column, while the protein and hydrophilic fraction of the sample was eluted to the waste. After a period of 5 min, the mobile phase composition and direction was switched to eluate the extracted benzodiazepines onto the analytical column for separation and UV detection. The presence of organic modifier in the extraction mobile phase was also important in achieving release of the protein-associated drugs, like benzodiazepines, from the protein complex [28]. Since most drugs are reversibly bound to serum proteins such as albumin via several lower affinity sites [29], a

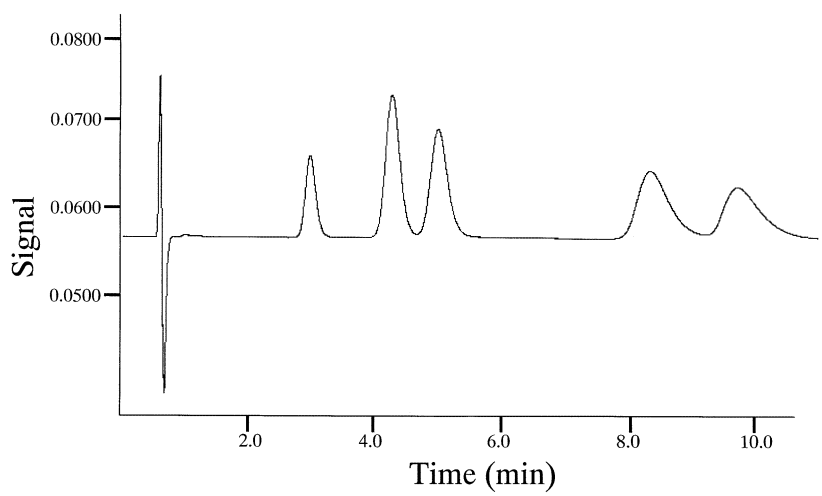


Fig. 4. Reverse-phase chromatographic separation of five benzodiazepines in water. Supelcosil C18 column (5.0 cm × 4.6 mm i.d.; 5 μm particle size); mobile phase, water–methanol (54:46 v/v); flow rate, 1.0 ml/min; sample injection volume, 20 μl; detection, $\lambda = 230$ nm.

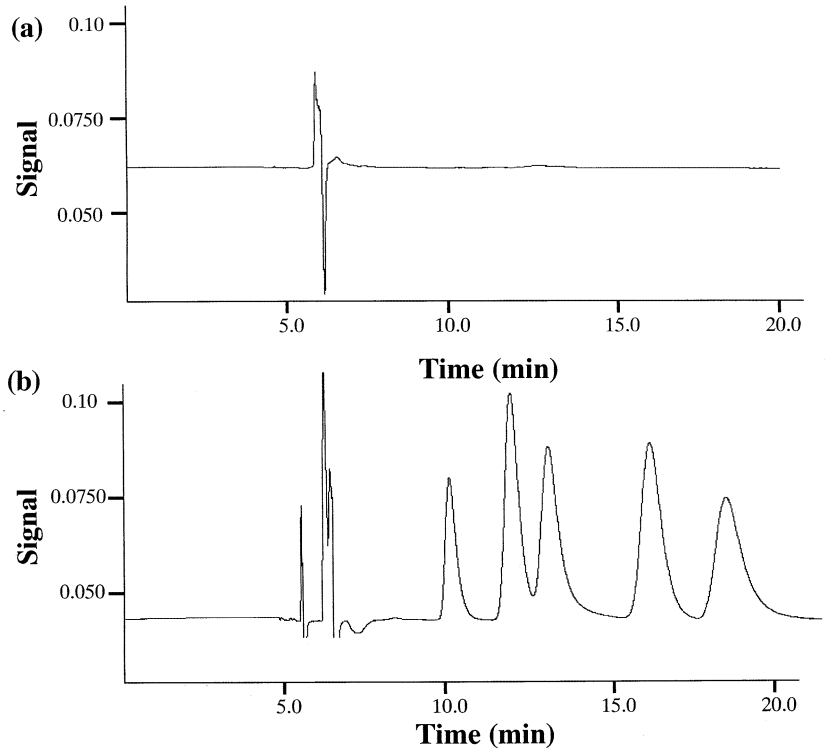


Fig. 5. (a) Blank serum chromatogram using the ADS-HPLC system. (b) Chromatographic separation of five benzodiazepine-spiked serum samples using the ADS-HPLC system: 100 μl sample loaded onto LiChroCART® 25–4 mm LiChrospher® RP-18 ADS, 25 μm extraction column with water–methanol (95:5 v/v); elution and separation, water–methanol (54:46 v/v) using a Supelcosil C18 column (5.0 cm × 4.6 mm i.d.; 5 μm particle size); flow rate, 1.0 ml/min; detection, $\lambda = 230$ nm.

small percentage of an organic solvent like methanol has been shown to effectively displace any bound drug [25,30]. Lipophilic compounds, like benzodiazepines [31], will also have a large interaction with the C18 stationary phase of the ADS column, furthering the dissociation of the drug–protein complex.

Fig. 5(a),(b) represents a typical chromatogram for both the blank and benzodiazepine-spiked serum sample. In both instances, the UV absorbance of the eluting protein fraction was not recorded. As shown in Fig. 5(b), the successful extraction and elution of the benzodiazepines from the ADS column, followed by separation of all compounds in the serum sample by the analytical column, was possible using a direct 100 μl injection. Preconcentration of the analytes on the ADS column allowed larger volumes to be injected than could be normally handled by a HPLC analytical column. The total cycle time was 20 min; however, this value could be shortened as the reconditioning of the ADS precolumn and the next injection can be performed simultaneous with the last injection's elution. The presence of sample carryover from the previous sample was evaluated by the injection of a blank serum sample after performing many spiked serum samples. As shown in Fig. 5(a), the non-appearance of peaks in the blank sample chromatogram confirms the absence of interferents resulting from analyte carryover or other interfering compounds in the serum sample. Blank urine chromatograms were also recorded and produce very similar results. Since no sample preparation was performed, the developed method was specific, as it was able to measure the benzodiazepine's response in the presence of all potential sample components.

Although the separation of the analytes was more than adequate for quantification purposes, when compared with the separation of benzodiazepine standards (prepared in water) on the C18 analytical column, the resolution of oxazepam and temazepam was slightly reduced at a resolution value of 1.2. There are several explanations for the observed peak broadening, such as extra column and tubing dead volume in the ADS-HPLC system. However, a more likely reason resulted from the elution efficiency of the extracted analytes from the ADS column. It is important to ensure the extracted compounds are eluted in as narrow a band as possible to prevent peak broadening. In addition to the changing the elution strength of the mobile phase, experimental parameters such as pH and increasing column temperature can be employed to enhance the elution efficiency.

The average recovery of the compounds from spiked samples, calculated by comparing the obtained peak area with those of aqueous solutions, was greater than 95%. Calibration curves were constructed, in triplicate, over a range of 0.05–10 μg/ml for the five compounds. As presented in Tables 2 and 3, excellent linearity was observed for all benzodiazepines in both serum (average $R^2 = 0.9888$) and urine (average $R^2 = 0.9948$) matrices with a calculated percent relative standard deviation (%RSD) of 3.9% for the slope value and 6.2% for the intercept value. The limit of detection (LOD) and limit of quantification (LOQ) for each compound in serum and urine was determined at a concentration where the signal/noise ratio was equal to 3 and 10, respectively, and these calculated concentrations have been included in Tables 2 and 3. The metabolism of the clinically prescribed benzodiazepines, such as diazepam and oxazepam into temazepam and nordiazepam, in addition to various dosage regimes, results in wide serum/urine concentration ranges being reported in the literature. However, for a typical 15 mg oral dose of oxazepam, concentrations as low as 50 ng/ml were reported in one patient's serum, while diazepam concentrations as high as 1050 ng/ml have detected in another patient's serum sample [32]. The detection limit of the developed ADS-HPLC method falls below the required detection limit for serum analysis and the linear dynamic range was an order of magnitude larger than required.

The reproducibility of the developed method was determined with ten injections of a 0.5 μg/ml sample. The injection repeatability was calculated as the %RSD for each benzodiazepine HPLC peak area in both the serum and urine, and the average value for all compounds in both matrices was determined to be 4.6%. The intra-assay precision was determined with repeated analysis of a sample that has been independently prepared, over 1 day, yielding an average %RSD of 4.3%. Several analysts have confirmed these results over a span of many weeks, indicating the ruggedness of the technique.

The applicability of the developed method was further validated with a clinical sample. A sample of urine was obtained from a volunteer, 12 h after receiving a 5-mg dose of diazepam. The major metabolite of diazepam in urine, temazepam, was detected in the urine and the recorded HPLC

Table 2
Linear regression data for benzodiazepine serum calibration curves

| Compound | Regression line[a] | | | Limits (ng/ml) | |
|---|---|---|---|---|---|
| | Slope | Intercept | $R^2$ value | LOD | LOQ |
| Clonazepam | 109 806 | 20 953 | 0.9743 | 38.8 | 129.3 |
| Ozazepam | 292 267 | −14 775 | 0.9988 | 24.2 | 80.7 |
| Temazepam | 316 141 | 4578 | 0.9779 | 31.7 | 105.7 |
| Nordazepam | 369 798 | −8408 | 0.9962 | 31.3 | 104.3 |
| Diazepam | 325 601 | −19 675 | 0.9968 | 45.0 | 150.0 |

[a] Concentration range, 0.05–10 μg/ml; number of data points, 8.

Table 3
Linear regression data for benzodiazepine urine calibration curves

| Compound | Regression line[a] | | | Limits (ng/ml) | |
|---|---|---|---|---|---|
| | Slope | Intercept | $R^2$ value | LOD | LOQ |
| Clonazepam | 133 054 | −21 972 | 0.9938 | 48.4 | 161.2 |
| Ozazepam | 327 633 | −23 242 | 0.9993 | 24.5 | 81.5 |
| Temazepam | 283 085 | 48 150 | 0.9958 | 31.7 | 105.7 |
| Nordazepam | 386 834 | −44 086 | 0.9977 | 33.1 | 110.3 |
| Diazepam | 343 562 | −63 933 | 0.9875 | 52.9 | 176.3 |

[a] Concentration range, 0.05–10 μg/ml; number of data points, 8.

peak area compared with the spiked urine calibration curve to yield a concentration of 67.9 ng/ml. Determination of this benzodiazepine in urine, by the ADS-HPLC method, was within the range previously reported [33].

The stability and robustness of the method were investigated by varying the percentage of methanol in the extraction and elution mobile phases. Adequate extraction and separation of each benzodiazepine compound from the serum and urine matrix was still possible with $\pm 5\%$ methanol in the mobile phase. However, as previously mentioned, too much organic content in the mobile phase during the extraction step can lead to irreversible protein precipitation on the column. The stability of the ADS extraction column has been previously reported using similar experimental conditions and was shown to be functional for over 2000 injections of a 50 μl injection of plasma [34]. Finally, the presence of a guard column in front of the analytical column maintains the lifetime of the analytical column, ensuring an overall stable and robust developed method for the determination of a many benzodiazepine compounds in biological samples.

## 4. Conclusions

The ADS extraction column provided a simple and on-line approach for the direct injection and extraction of benzodiazepines in complicated matrices, such as serum and urine. In comparison with existing procedures for benzodiazepine determination, the procedure was simple, easily automated and, most importantly, decreased the overall sample analysis time since there was no requirement for sample preparation. A decrease in sample preparation also provides safer handling of potentially bio-hazardous material. Enhancements in the optimization time required for the ADS-HPLC method development was possible with a correlation between the analyte's partition coefficient on the ADS column and its $\log P$ value, providing a further improvement in this procedure. Column and mobile phase switching was employed for the desorption, separation and UV determination of the extracted benzodiazepine compounds under isocratic and non-buffered solvent conditions, providing additional robustness to the method as it eliminates the requirement for the preparation of more complex mobile phases. Preconcentration from large volume injection provided suitable detection limits ($< 50$ ng/ml) for routine analysis demonstrating the robustness and suitability of the ADS extraction column for the determination of this class of clinically significant compounds.

The isolation and separation of analytes can be further simplified with the modification of the interior extraction phase to render the sorbent more selective [35] and the use of restricted access materials even more versatile.

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