CHAPTER 6
ANALYSIS OF DIAZEPAM USING HIGH PERFORMANCE THIN LAYER CHROMATOGRAPHIC METHOD

Introduction:

Diazepam is a drug belonging to benzodiazepine group (7-chloro-1-methyl-5-phenyl-3H-1,4-benzodiazepine-2-one) known for its depression activity on the central nervous system [M. Abid et al., 2006] with anticonvulsant, anxiolytic, sedative and muscle relaxant properties [J. P. Hullihan et al., 1983]. It is also used in the treatment of alcoholic withdrawal syndrome [M. Lucht et al., 2003] and in the treatment of organophosphorus poisoning [T. C. Marrs, 2003]. Diazepam is one of the most widely prescribed benzodiazepine for a variety of conditions particularly for anxiety, depression, epilepsy and insomnia [M. Abid et al., 2006, L. P. Longo and B. Johnson, 2005]. Diazepam was first synthesized in 1955 by Leo Sterbatch [Uchibayashi. M, 2007, Tauseef Mehdi, 2012] and marketed by Hoffmann-La Roche in 1963 in the brand name as Valium [Tauseef Mehdi, 2012]. The mechanism of action of diazepam is attributed to the production of the neurotransmitter gamma-aminobutyric acid (GABA) [Riss J et al., 2008]. The synthesis, chemical neuroscience, pharmacology, drug metabolism, adverse effects, dependence, clinical use and regulatory issues were all discussed by Nicholas E. Calcaterra and James C. Barrow [Nicholas E. Calcaterra and James C. Barrow, 2014]. Diazepam in clinical dosage form as valium when given as therapy to patients proved to improve health conditions and enabled the patients to think more clearly [H. Marlin Beerman, 1964]. Clinical vigilance regarding withdrawal of benzodiazepines after a long term prescription was urged for all consumers especially important among alcoholics consuming high doses of benzodiazepines [P. Cushman and D. Benzer, 1980]. Diazepam
and its metabolites are useful in management of disturbed sleep [Coral H. Clarke and A.N. Nicholson, 1978]. Also there was evidence that shows increased crash risk in drivers who were using benzodiazepines like diazepam for some anxiety disorder [M.C. Longo et al., 2001]. In cases of overdose of diazepam or other benzodiazepines [Geoffrey K Isbister et al., 2004, B. Ukley and W. Hermann, 2012, C.Oti et al., 2010] it may be necessary to carry out the analysis and confirm the concentration of the same in the victim’s blood. Therefore, sensitive methods for the determination of benzodiazepine are considered to be necessary. Changes in plasma concentrations of diazepam with different ways of administration was reported in literatures [D.W.Sturdee, 1976] for which, it is essential to adopt suitable method for analysis of the same.

When literature was surveyed it was found that using HPLC alone, there were many methods available for quantitative determination of diazepam from various biological and non biological samples using different procedures. They include; extraction of diazepam from rat’s brain with SPE method [Mercolini L et al., 2009], analysis of diazepam and its metabolites from human blood [Kabra PM et al., 1978], in human plasma and urine [Azzam RM et al., 1998], extraction using ether from the blood treated with aqueous ammonia [H.M.Stevens, 1985]. To detect over dosages and also to determine therapeutic dosages, from serum diazepam using GC, chloroform extraction was used [Lowell B. Foster and Christopher S. Frings, 1970]. Diazepam in pharmaceutical preparations was quantified using HPLC with RP column in dosage forms [M.Lazar et al., 2013], in tablet form along with propranolol hydrochloride with RP system [Patel Satish A et al., 2011], in combined dosage forms along with imipramine hydrochloride [P.Pydiratnam et al., 2013, VRB Vemula and PK Sharma, 2013] and from
injectable formulation together with degradation product, 2-methylamino-5-chlorobenzophenone [F. M. Smith and N.O. Nuessle, 1982]. FTIR spectroscopic method is also available for the quantification of diazepam in pharmaceuticals by extracting with chloroform and measuring the area of the peak in the interval 1672-1682 cm$^{-1}$[Javier Moros et al., 2006]. Similarly, among spectrophotometric methods, diazepam was treated with alkaline dimethyl sulphoxide which produces reddish colour because of the formation of charge transfer complex [Mohammad Sarwar, 2008]. Another method of quantification of diazepam involved 1:1 ion-association complex formation with bromocresol green at pH 3.5 and extraction of the dye into chloroform layer followed by spectrophotometric measurements [S.Sadeghi et al., 2002]. With the help of derivative spectroscopy diazepam was quantified in microemulsion gels in presence of propylene glycol and Tween 80 [D.G.Dastidar and Biswanth Sa, 2009] and in presence of otilonium bromide [Mannucci C et al., 1992]. Other simple methods include interaction of diazepam with picric acid dinitrobenzoic acids to form products which is used for spectrophotometric determination for some pharmaceutical preparations [W.F.El Hawary et al., 2007]. Rapid Resolution Liquid Chromatography-Tandem Mass Spectrometry for some benzodiazepines including diazepam and its metabolites has been reported for clinical urine samples [Ren-Yu Hsu et al., 2013]. Molecularly imprinted solid phase extraction protocol for estimation of diazepam from hair of postmortem samples has been compared with SPE LC MS MS method [M.M.Ariffin et al., 2007]. Diazepam along with its metabolites was determined from the hair samples of abusers using GCMS [Sooyeun Lee et al., 2011]. Gas Chromatography was used for measurement of diazepam and its metabolites in plasma samples using ECD [W. Loscher, 1982] and in whole blood using
GC with NPD detector with derivatization process [Horton Mccurdy et al., 1979]. Cerebrospinal fluid was used as an alternate testing matrix for monitoring concentration of diazepam when blood was not available for analysis using GC MS was presented by David A. Engelhar and Amanda J. Jenkins [David A. Engelhar and Amanda J. Jenkins, 2007]. Electrochemical quantification of diazepam in pharmaceutical products using polarographic studies was also presented [M.G. Garcia et al., 1993]. Electrochemical behavior of diazepam at the modified carbon paste electrode was done using cyclic voltammetry and square voltammetry methods which gave linear range and very low detection limits, successfully applied for the determination of diazepam in tablets and human serum [M.R. Milani Hosseini and Ali Motahrian, 2015]. The methods for the determination of diazepam from human plasma / blood involves the use of special techniques like solid phase microextraction coupled with LC MS [M.Walles et al., 2004] or coupled with GC MS [M.H.De Oliveira et al., 2005].

Thin Layer Chromatography (TLC) is basically used to monitor reaction products, purity of the sample, active ingredient quantification [Naveen Bimal and Bhupinder Singh Sekhon, 2013]. By applying spots on plates, usually made of silica gel as stationary phase, the solvent was made to move on the stationary phase through capillary action by keeping the stationary phase inside a mobile phase it was depending on the polarity of solvent, the constituents of the spot start to separate. High Performance Thin Layer Chromatography (HPTLC) is an enhancement over TLC as the developed plates can be scanned under UV light and the corresponding spectrum of each separated spot can be obtained. In HPTLC, simultaneous multisampling analysis on a single plate allows a real in system calibration in contrast to other chromatographic techniques [P.D.Sethi,
HPTLC was preferred over HPLC in some studies due to simplicity, ease and low cost [M.Bakavoli and M.Kaykhali, 2003]. HPTLC is proven to be a very useful instrument for analysis of many types of samples in many areas [Naveen Bimal and Bhupinder Singh Sekhon, 2013].

A number of HPTLC methods are also available for quantification of diazepam from cold drinks which are adulterated for criminal motives [R.K.Sarin et al., 1998], extracting the sample at pH 8.5 with diethyl ether and chloroform and scanning the developed spots at 230 nm. Diazepam in tablets forms were analyzed with HPTLC by extracting the active ingredient into methanol and densitometric measurement at 232 nm using toluene and acetone as mobile phases [V.P.Machale et al., 2011]. HPTLC analyses of diazepam along with imipramine using the zero crossing point and derivative spectra were used for quantification from combined dosage form [Bhadani Shweta et al., 2013]. Using chloroform-isopropanol mixture, the acid hydrolyzed product of diazepam was extracted from urine samples and it was quantified with HPTLC [R.Jain, 1993]. By spraying with a reagent of sodium hydroxide and m-dinitrobenzene in dimethyl sulfoxide, amongst other benzodiazepines, it was found that only diazepam produces violet bands on TLC plates in biological samples [B.B. Daundkar et al, 2008]. In another study by P.K.Salo et al, diazepam along with other benzodiazepines were separated with dichloromethane and acetone (93:7) as one-dimensional mobile phase, toluene, acetone, ethanol and 25% ammonia (70:20:3:1) for two dimensional separation, followed by detection at 254 nm [P.K.Salo et al., 2007]. In most of the earlier works, solvent systems used for TLC of diazepam involve the use of more harmful solvents like toluene or chloroform as mobile phases.
Liquid-liquid extraction is the most commonly used method for extraction of drugs from the matrices. Diazepam together with other drugs were extracted with less toxic solvents from spiked blood samples using chlorobutane, ethyl acetate, diethyl ether, heptane, hexane and iso-octane solvents [ZhiBin Huang et al., 2015]. Extraction of diazepam was carried with diethyl ether from blood [Haram K et al., 1976], with chloroform from serum [Lowell B. Foster and Christopher S. Frings, 1970], with toluene [Raisys VA, 1980] and with benzene from plasma [Loscher, 1982] have been described. As a consequence of literature on diazepam [M. Abid et al, 2006, L.P. Longo 2005, J. P. Hullihan et al., 1983] and considering the importance of diazepam analysis in various samples [H. Marlin Beerman, 1964, P. Cushman, 1980, Geoffrey K Isbister et al., 2004, B. Ukley et al., 2012, C.Oti et al., 2010], it is here, efficacy of diazepam extraction from blood samples is performed by HPTLC experiments using three different extraction solvents namely, acetonitrile, chloroform and diethyl ether was checked in neutral, acidic and basic conditions and determined diazepam using new HPTLC method employing an optimized mobile phase, hexane and ethyl acetate (7:3).

The developed new HPTLC method with and hexane ethyl acetate (7:3) was successfully applied for the determination of diazepam present in forensic blood samples and the samples of seized drug material and also present in some pharmaceuticals. Diazepam was shown to be very stable in blood and tissues even for several months under room temperature [B. Levine et al., 1983] and so blood was chosen to spike with diazepam for its determination through the new method [Vani. N et al., 2013].
Objectives:

To develop new HPTLC method involving hexane and ethyl acetate (7:3) as solvent system for the determination of diazepam present in blood and pharmaceutical samples.

Experimental:

Materials and Methods:

Instrumentation: HPTLC system (Camag, Muttenz, Switzerland) fitted with auto sampler, UV scanner and photo documentation system - loaded with Wincats software.

UV-visible double beam spectrophotometer (Chemito, India) and Fourier Transform Infra Red spectrometer (Thermo Scientific, USA) were used.

Materials: Diazepam used here was recrystallised in methanol and purity was checked with its melting point, UV and IR spectral analysis. AR grade acetonitrile, methanol, chloroform, diethyl ether, Silica gel 60 F_{254} TLC plates (E.Merck) were used. The blood sample used here was obtained from a local hospital.

Preparation of standard diazepam solution:

Standard stock solution of 1 mg/mL diazepam was prepared by dissolving 0.1 g of diazepam in methanol and diluting the solution to 100 ml with methanol. This solution was diluted or used as such to get different concentration of diazepam, used in calibration and spiked blood sample preparations.

Extraction procedure for sample preparation:

2 ml blood sample was spiked with different volumes of diazepam stock solution and each sample was vortex mixed for 5 min. Extraction was carried out thrice with 5 ml of acetonitrile, centrifuged at 3000 rpm for 15 min., filtered through Whatmann 40 filter
paper containing silica and the filtrate contained in a beaker was evaporated on a water bath maintained at 60°C. The residue remained was extracted with methanol and transferred the extract into a 5 ml in a volumetric flask and diluted to the volume with methanol. An aliquot of this was transferred into a labeled HPTLC glass vial and spotted on the TLC plate so as to get 5 µg/spot. Similar extractions were carried out by making spiked blood acidic with 1 ml 0.01M hydrochloric acid (pH 2), basic with 1 ml 0.1M ammonia (pH 11). The same procedure was repeated with chloroform and diethyl ether instead of acetonitrile. Blank blood samples without diazepam were processed in the same way and it revealed a profile with negligible interference.

**HPTLC method and chromatographic conditions:**

The labeled vials with different concentration of diazepam solutions were kept in Camag auto sampler ATS 4 and known volumes were spotted (band length, 6 mm) on the plates along with different volumes of standard solution using the Wincats software. These plates, that were washed with methanol and dried at 80°C before application of the samples, were developed in a solvent chamber, previously saturated with hexane and ethyl acetate (7:3) as mobile phase. Each plate was developed to a distance of 80 mm, removed from the chamber, solvent was removed by drying with hot air and developed spots on the plate was digitally documented using in built Camag documentation system with camera by illumination at 254 nm.

After documentation, quantification was done with densitometric measurement at 230 nm followed by UV scanning with Camag TLC scanner 3. Using the following parameters the densitogram (chromatogram) and the UV spectrum were recorded.

**HPTLC instrument parameters**
Method validation:

The following parameters were used to validate the developed HPTLC method.

Linearity and range:

From the stock solution of diazepam, appropriate dilutions were made to get a series of solutions with concentrations ranging from 0.01 to 1 mg/mL. 20 μL of these prepared solutions were spotted on three different plates so as to get a series of spots having the concentration range, 0.2 - 20 μg/spot. Plates were developed as described earlier and average peak areas were plotted against the corresponding concentrations of diazepam to get its calibration curve and linear regression equation.

Limit of Detection (LOD) and Limit of Quantification (LOQ):

LOD was calculated as the concentration at which signal to noise ratio was more than three and LOQ was calculated as signal to noise ratio was more than ten.

Precision:

2.5, 1.25 and 0.5 ml of 1 mg/mL stock solution was added to 2 ml blood samples. Extractions were carried with diethyl ether by making the solutions basic and reconstituted in 5 ml methanol as explained earlier. The intra-day repeatability was evaluated by spotting, 20 μL of each of the above solutions three times repeatedly on the plates and then plates were scanned to know the peak areas after development in mobile phase. Similarly inter-day reproducibility was evaluated by five times analysing these
sample solutions over a period of three days. Amount of diazepam was estimated by applying the obtained peak area values to the regression line equations. Accuracy of the method is reported as percent recovery and error.

**Specificity:**

In order to understand the interference for the determination of diazepam by new HPTLC method from some commonly used benzodiazepines with diazepam, equal volumes of 1 mg/mL solution of nitrazepam, chlonazepam, lorazepam, chlordiazepoxide, alprazolam and clozapine were added with 1 mg/mL solution of diazepam, one at a time and spotted on the TLC plate and developed it using the new mobile phase as discussed earlier and they were individualized by calculating the Rf and UV pattern.

**Results and discussion:**

**Selection of mobile phase for thin layer chromatography:**

Ethyl acetate together with ammonium hydroxide, methanol and with acetonitrile were used as mobile phases for the quantification of diazepam from pharmaceuticals [Bernard Fried and Joseph Sherma, 2005]. Frequently used benzodiazepine derivates including diazepam were separated using Stahl’s triangle based on structure, polarity, solubility and stability concepts to choose mobile phases after in situ hydrolysis to form benzophenones [Hancu G et al., 2011]. Similarly, Subbiah Thangadurai et al. have considered chloroform and methanol (97:3) as a good solvent system for the separation of nine benzodiazepines among chosen ten solvent systems [Subbiah Thangadurai et al., 2013] using TLC. By considering the toxic nature of toluene and chloroform mobile phases used in earlier works for benzodiazepines [M.Bakavoli and M. Kaaykhalil, 2003,
V.P. Machale et al., 2011, B.B. Daundkar et al., 2008], a new solvent system consisting of hexane and ethyl acetate was developed here for diazepam to separate it from other benzodiazepines as ethyl acetate was considered as greener solvent [Yao Shen et al., 2015]. A mixture of these solvents was used in different proportions and a fraction of 7:3 v/v of hexane and ethyl acetate was giving a good separation with reproducible results (SD = 0.09) having retention factor 0.34 (Rf = distance traveled by analyte/distance traveled by mobile phase). This mobile phase was used for further studies.

![Graph](image1.png)

**Fig. 1:** Variation of retention factor with change in fraction of hexane and ethyl acetate

After development with the mobile phase, the plates were viewed under UV light which shows the black coloured spots in green background and the image was captured with Camag Reprostar 3, photo documentation system. Commercially available TLC plates are normally coated with fluorescent quenching materials so as to facilitate UV detection [Caitlin Sullivan and Joseph Sherma, 2004]. The photo captured one such plates and subsequently sprayed with Iodoplantine reagent which gives blue violet colour spots [Lukasz Komsa et al., 2014] are shown in Fig. 2. Overlaid densitograms for the developed spots used in making calibration curve and their corresponding overlaid UV spectra are shown in Fig. 3.
Fig. 2: Developed plate with photographed under UV light (left) and same plate after UV scanning was sprayed with Iodoplatinate reagent (right) which gives violet coloured spots for diazepam

Fig. 3: 3D display of densitogram of developed spots of diazepam on the TLC plate showing Rf =0.34 (left) with inlay indicating concentration of each densitogram and their overlaid UV spectra (right) with λ max = 233 nm

Selection of solvent for extraction:

The efficacy of extraction and percentage recoveries of the diazepam with three different solvents and each solvent at three different pH conditions were experimented for an intermediate concentration of diazepam, 5 μg/spot, the results obtained are given in Table 1.
Table 1: Efficacy of extraction with different solvents

<table>
<thead>
<tr>
<th>Solvent</th>
<th>pH</th>
<th>Filtration speed</th>
<th>Precipitation of blood</th>
<th>Filtrate</th>
<th>Recovery %</th>
<th>SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acetonitrile</td>
<td>2</td>
<td>Fast</td>
<td>Yes</td>
<td>No need for separation</td>
<td>92.0</td>
<td>0.81</td>
</tr>
<tr>
<td></td>
<td>7</td>
<td>Slow</td>
<td>Yes</td>
<td></td>
<td>91.3</td>
<td>1.15</td>
</tr>
<tr>
<td></td>
<td>11</td>
<td>Fast</td>
<td>Yes</td>
<td></td>
<td>89.4</td>
<td>0.72</td>
</tr>
<tr>
<td>Diethyl ether</td>
<td>2</td>
<td>Fast</td>
<td>Slight</td>
<td>Needs separation</td>
<td>84.6</td>
<td>0.55</td>
</tr>
<tr>
<td></td>
<td>7</td>
<td>Fast</td>
<td>No</td>
<td></td>
<td>96.4</td>
<td>1.42</td>
</tr>
<tr>
<td></td>
<td>11</td>
<td>Fast</td>
<td>No</td>
<td></td>
<td>98.6</td>
<td>0.95</td>
</tr>
<tr>
<td>Chloroform</td>
<td>2</td>
<td>Slow</td>
<td>Slight</td>
<td>Needs separation</td>
<td>88.6</td>
<td>1.15</td>
</tr>
<tr>
<td>Ref. method [St. Pierre MV, 1987Using ethyl acetate]</td>
<td>7</td>
<td>Slow</td>
<td>No</td>
<td>Needs separation</td>
<td>90.1</td>
<td>0.85</td>
</tr>
<tr>
<td></td>
<td>11</td>
<td>Slow</td>
<td>No</td>
<td>Needs separation</td>
<td>92.5</td>
<td>0.97</td>
</tr>
<tr>
<td></td>
<td>7</td>
<td>Slow</td>
<td>No</td>
<td>Needs separation</td>
<td>94.4</td>
<td>0.92</td>
</tr>
</tbody>
</table>

Acetonitrile is used as a deproteinization reagent for the extraction of benzodiazepines from whole blood [Jessica L. Westland and Frank L. Dorman, 2013]. When acetonitrile was used as a solvent for the extraction of diazepam from the blood sample, the blood was precipitating with the solvent and that enabled the filtration easier and neat. But acetonitrile was miscible with water which makes serum to get mixed with the filtrate, which was taking a longer time for the evaporation of the filtrate containing diazepam. Further, extraction of diazepam from the blood sample using acetonitrile solvent was yielding lower recoveries of diazepam which may be due to its trapping in the precipitate (B.Levine, 1999) and it needed more volume of the solvent than the specified 15 ml for its effective recovery. However, when diazepam was extracted with acetonitrile from the blood sample that was made acidic was giving a good recovery compared to the samples which were basic or normal.
When the solvent chloroform was used for the extraction of diazepam from the blood sample, there was a formation of emulsion that was making the process of extraction and filtration difficult. Recovery of diazepam into chloroform from the spiked blood samples at three different pH conditions was found to be less compared to its extraction either into acetonitrile or diethyl ether. However, diazepam extraction into chloroform from the blood sample which was made acidic was less compared to the blood samples that were normal and made basic.

When the solvent diethyl ether was used to extract diazepam from the blood sample that was made acidic there was a slight precipitation but such precipitation was not observed when attempted to extract the diazepam either from the normal blood sample or the sample that was made basic. As the solvent diethyl ether was evaporating quickly from the filtrate, time needed for its removal was less than 5 min on a water bath at 60° C. There was a good diazepam extraction in to diethyl ether either from the normal blood sample or from the sample that was made basic but its extraction from the sample that was made basic was better. Diethyl ether was proved as a solvent of choice for extraction of diazepam from fermented fruit juices [Lukasz Komsta et al., 2013] and by considering the less toxic nature of diethyl ether, in the present study also, diethyl ether has been used. This extraction process of diazepam was not involving more number of extraction steps and also unlike other methods that involve special techniques [M.Walles et al., 2004, M.H.De Oliveira et al., 2005]. A good recovery of diazepam, 98.6% (%RSD=1.13) was an indication that the method developed here could be used in the new HPTLC method for the accurate and quantitative determination of diazepam from the blood samples.
Further, the method was compared with another thin layer chromatographic procedure using ethyl acetate as extraction solvent and developed in a solvent chamber consisting of chloroform-ethyl acetate-ethanol-ammonium hydroxide [St. M.V. Pierre, 1987], followed by densitometric measurement at 230 nm. Even though, a good recovery of diazepam was obtained, 94.4% (%RSD=0.92), the spot of diazepam will move almost to the solvent front (Rf=0.97) which makes it difficult to scan the spots since we get a zig-zag pattern near the solvent front due to ammonium hydroxide, indicating better validity of hexane and ethyl acetate (7:3) system.

**Validation parameters:**

The validation parameters established for the quantitative determination of diazepam through the new method are given in Table 2. The relationship between the concentration of diazepam in standard solutions and the corresponding peak responses at 230 nm was found to be linear in the range of 0.5-20 μg/spot of diazepam with correlation coefficient of 0.998. The results obtained are shown in Fig.4. The values of LOD and LOQ were found to be 0.2 and 0.5 μg/spot respectively.

**Table 2: Summary of validation parameters**

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Linearity range</td>
<td>0.5 – 20 μg/spot</td>
</tr>
<tr>
<td>Correlation coefficient</td>
<td>0.998</td>
</tr>
<tr>
<td>Slope</td>
<td>32.19</td>
</tr>
<tr>
<td>Intercept</td>
<td>154.4</td>
</tr>
<tr>
<td>Limit of detection</td>
<td>0.2 μg</td>
</tr>
<tr>
<td>Limit of quantification</td>
<td>0.5 μg</td>
</tr>
</tbody>
</table>
Fig. 4: HPTLC calibration graph of diazepam

Precision and repeatability for the determination of diazepam in its three different concentrations as indicated in Table 3 for spiked human blood sample extraction using diethyl ether in basic media are reflecting a good robustness of the method.

Table 3: Accuracy and precision data of diazepam in spiked human blood

<table>
<thead>
<tr>
<th></th>
<th>Actual Concentration [μg]</th>
<th>Measured concentration [μg]</th>
<th>Recovery [%]</th>
<th>SD [μg]</th>
<th>RSD [%]</th>
<th>Accuracy Error [%]</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Intra-day</strong>a)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(n=3)</td>
<td>2</td>
<td>1.97</td>
<td>98.06</td>
<td>1.11</td>
<td>1.13</td>
<td>-1.5</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>4.84</td>
<td>96.32</td>
<td>0.91</td>
<td>1.50</td>
<td>-3.2</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>9.76</td>
<td>96.24</td>
<td>1.44</td>
<td>1.50</td>
<td>-2.4</td>
</tr>
<tr>
<td><strong>Inter-day</strong>b)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(n=3)</td>
<td>2</td>
<td>1.89</td>
<td>94.83</td>
<td>3.88</td>
<td>4.10</td>
<td>-5.5</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>4.75</td>
<td>95.13</td>
<td>3.93</td>
<td>4.13</td>
<td>-5.0</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>8.98</td>
<td>89.86</td>
<td>5.46</td>
<td>6.08</td>
<td>-10.2</td>
</tr>
</tbody>
</table>

a) mean for n =3  
b) mean for n = 5

**Evaluation of specificity of the new mobile phase for diazepam:**

In addition to diazepam there are other benzodiazepines that were reported to be most commonly prescribed (L.P. Longo, 2000). Therefore, workability of the new solvent system for estimation of diazepam in presence of such benzodiazepines was determined.
**Fig. 5**: UV spectra of benzodiazepines separated using the proposed mobile phase

[a]: UV spectrum of alprazolam with $R_f = 0.02$ and $\lambda_{max} = 227, 249, 285$ nm

[b]: UV spectrum of nitrazepam with $R_f = 0.05$ and $\lambda_{max} = 278, 308, 225$ nm

[c]: UV spectrum of clozapine with $R_f = 0.08$ and $\lambda_{max} = 233, 262, 295$ nm

[d]: UV spectrum of lorazepam with $R_f = 0.1$ and $\lambda_{max} = 237, 257, 325$ nm

[e]: UV spectrum of clonazepam with $R_f = 0.13$ and $\lambda_{max} = 306, 260, 222$ nm

[f]: UV spectrum of chlordiazepoxide with $R_f = 0.03$ and $\lambda_{max} = 258, 316$ nm

[g]: UV spectrum of diazepam with $R_f = 0.34$ and $\lambda_{max} = 233, 254, 310$ nm
Each sample was individualized by UV pattern and Rf values as given in Fig. 4. The results obtained are accounting for the effective separation of diazepam from other benzodiazepines. Therefore, diazepam could be determined quantitatively even when it is present along with other benzodiazepines.

Applications:

1. Postmortem blood sample: Earlier discussed method was employed to extract diazepam from 2 ml of post mortem blood sample using diethyl ether in basic medium and the extract was spotted on the TLC plate and developed with the new mobile phase. A concentration of 2.981 µg/ml of blood was obtained with the new method which was sufficient to produce toxic effects but it was less for causing fatalities [E.G.C. Clarke, 1986], but there was also a smothering effect as reported by the doctor in his post mortem report.

2. Forensic drug sample: A white color powder sample was received to the laboratory for the analysis of diazepam. A known quantity of the sample was extracted in methanol and an aliquot was spotted on the TLC plate and quantified using the new mobile phase. An average concentration of only 5.6% of diazepam was obtained and the remaining material was being urea/other adulterants.

3. Pharmaceutical samples: The new method was applied to determine the diazepam present in four different market samples; three samples were with single component and the other one was with double components. Imipramine hydrochloride is a benzodiazepine with antidepressant property which can be effectively separated from diazepam with respect to Rf and UV absorption pattern (Rf = 0.05 and λ max = 251 nm). Known amount of the values obtained were tabulated in Table 5. The results indicate
that the new HPTLC method was simple and specific for the estimation of diazepam in its formulations.

**Table 4: Analysis of diazepam in dosage forms**

<table>
<thead>
<tr>
<th>Sample</th>
<th>Components</th>
<th>Label claim (mg/tablet)</th>
<th>Amount found (mg/tablet)</th>
<th>% Assay(^a)</th>
</tr>
</thead>
<tbody>
<tr>
<td>01</td>
<td>Diazepam</td>
<td>2</td>
<td>1.94</td>
<td>97.0±0.06</td>
</tr>
<tr>
<td>02</td>
<td>Diazepam</td>
<td>5</td>
<td>4.92</td>
<td>98.4±0.05</td>
</tr>
<tr>
<td>03</td>
<td>Diazepam</td>
<td>10</td>
<td>9.88</td>
<td>98.8±0.16</td>
</tr>
<tr>
<td>04</td>
<td>Diazepam with Imipramine HCl</td>
<td>2</td>
<td>2.07</td>
<td>103.5±0.10</td>
</tr>
</tbody>
</table>

\(^a\) - mean ± standard deviation of five determinations

**Conclusion:**

HPTLC technique combined with sample application and densitometric scanning has been proved to be sensitive, reliable and suitable for qualitative and quantitative analysis of pharmaceutical, environmental, toxicological, forensic and food samples [P.K.Salo et al., 2007]. Therefore, in the present work, without using more toxic solvents like toluene and chloroform [M.Bakavoli and M. Kaaykhali, 2003, V.P.Machale et al., 2011, B.B. Daundkar et al., 2008], separation of benzodiazepines was carried out using ethyl acetate and hexane solvents. Hexane and ethyl acetate in the ratio (7:3 v/v) was found to be a very effective new mobile phase for HPTLC determination of diazepam with Rf value 0.34 (SD=0.09). Using different techniques, samples containing diazepam has been extracted using solvents like chloroform [Lowell B. Foster and Christopher S. Frings, 1970], toluene [Raisys VA et al., 1980] and with benzene from plasma [Loscher, 1982]. Therefore, an attempt was made here to develop a method of extraction of diazepam with diethyl ether in basic medium and it was found to be very specific without
the interference from common benzodiazepines [Vani. N et al., 2013]. As the results were reproducible, specific, less time consuming and simple without use of special techniques [M.Walles et al., 2004, M.H.De Oliveira et al., 2005], the method is suitable for practice in any clinical or forensic laboratory for the satisfactory determination of diazepam present in blood and pharmaceutical samples.

The results obtained from the study of optimization of extraction procedure, selection of solvent system and quantification of diazepam from spiked blood samples were published in JPC-Modern TLC, 26(4) (2013) 343-348.
Summary

A high performance thin layer chromatographic method combined with densitometric measurement and UV scanning was used to optimize new mobile phase consisting of hexane and ethyl acetate (7:3 v/v) for the estimation of diazepam in spiked human blood, forensic and pharmaceutical samples. Efficacy and ease of extraction with three solvents acetonitrile, diethyl ether and chloroform, each at three different pH conditions were studied. Validation parameters for diazepam were found to be linear in the range 0.5-20 μg/spot with r=0.998, LOD, 0.2 μg, LOQ 0.5 μg. Usefulness of the new mobile phase for the separation of six common benzodiazepines namely nitrazepam, clonazepam, lorazepam, chlordiazepoxide, alprazolam, clozapine from diazepam and were identified through their retention factor and UV pattern.
References:


[34] Ren-Yu Hsu, ShanAn Chan, Shu-Ling Lin, Tzuen-Yeuan Lin, Wei-Lan Chu, Ming-


