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SCIENTIFIC SECTION

Propofol injectables stability in the presence of midazolam. High-performance liquid chromatography avaluation

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Key words:
Stability, injectable mixtures, propofol, midazolam.

Summary:
In this study, the stability of injectable mixtures comprising 8mg/ml of propofol and 0,6 mg/ml of midazolam was tested. Previously, the physical stability of this association was demonstrated for a 24-hour period.
The concentration of both active principles was determinated in a range of 0 to 36 hours evaluating the contents of three prefilled syringes prepared simulta-neously at the start of the assay.

Propofol and midazolam were evaluated by two validated high-performance liquid chromatography techniques. After 12 hours perfusion for each of the three studied series, concentration of both drugs decreased aproximately 10%.

Perfusion periods longer than 24 hours produced, in vitro, losses in concentration highest than limits accepted in clinical practice and preparation of this treatment 36 hours in advance is not viable.

Introduction

The desire to optimize comfort in patients admitted to the intensive-care unit (ICU) has prompted the study of various sedative regimens and a global evaluation of the same with the goal of achieving ideal sedation.

Currently, the drugs most frequently utilized in this type of patient are propofol and midazolam[1]. Joint administration of these two drugs would be interesting from a clinical point of view, since such an association could offer a sixfold reduction in the drug doses necessary, both for the induction of anesthesia[2] and for maintaining sedation in ICU patients, as well as offering a reduced hemodynamic impact and lower costs[3].

We previously performed a study[4] that demonstrated the physical stability of this association for a 24-hour period and under conditions similar to those in wich the preparation is used (prefilled syringes with the mixture, samples taken from the end of a needle connected to an infusion system at previously established time intervals). From this study we concluded that extemporaneous preparation of the

mixture is unnecessary and, furthermore, that joint injection in patient presents no problems of a physical nature such as an increase in the drop size of the emulsion vehicle, sedimentation, etc.

Propofol is a short-acting intravenous anesthetic agent suitable for induction and maintenance of general anesthesia. It has previously been used in association with other drugs. Studies on its compatibility with other intra-venously administered drugs have been published[3,5,6].

Midazolam is a basic benzodiazepine with a relatively short half-life. It can be used to induce general anesthesia as a water-soluble salt formulation for administration as an intravenous solution in an acidic medium[7].
In this study we update and validate two high-performance liquid chromato-graphy (HPLC) techniques for the chemical evaluation of propofol and midazolam in joint injectable mixtures, with the goal of determining any possible chemical degradation of these active principles over time and under the ususal conditions of hospital administration.

From the experimental results, we try to obtain conclusions with regard to:
1. The possibility of preparing the studied mixtures nonex-temporaneously in order to facilitate operations in the hospital pharmacy.
2. The chemical stability of the mixture, in order to guarantee correct dosing of both active principles to patients throughout the perfusion period.

Methods

1.*Sample Preparation*
Mixtures of 400 mg propofol, corresponding to 40 ml Diprivan emulsion (Zeneca lot K14); 30 mg midazolam, corresponding to two 3-ml ampoules of Dormicum" (Roche lot K1), and 4 ml of water for injectables were prepared. The choice of the relative proportion of each drug in the mixture was based on sedative protocols used in the hospital environment. The mixtures were prepared under a laminar flow hood in order to ensure sterile conditions. Sterile 50-ml disposable syringes were filled with the mixtures.

2.*Sampling*
2.1 Device
Syringes were connected to a perfusion system and samples were taken from the tip of each (a 40 x 0.7 sterile hypodermic needle), wich was connected to a type-I 5ml glass vial stoppered with an elastomeric stopper. The needle base was connected to a PVA lengthener (ref. Hospal-hemoli-ne ES-1 673840 lot N9622) and the set to a 50ml syringe that was regulated to empty at 3 ml/hour by means of a perfusion pump (becton Dickinson Program 2).

3.*Assay Duration and Conditions*
The assay duration was 36 hours, a time that was equal to the emptying rate of three syringes that were all prepared simultaneously at the start of the assay.

The first sample was taken of the three simultaneously prepared syringes at t=0. One of the syringes underwent perfusion for 12 hours, during wich time a 3ml sample was collected every hour in a vial.

The second syringe was refrigerated for 12 hours and after was perfused with samples taken between t=12 hours and t=24 hours. The third syringe, was refrigerated for 24 hours and perfused with samples taken between t=24 and t=36 hours. A sample was taken before initiating perfusion, in all cases.

The contents of the vials were inmediatety frozen at –20 °C in order to avoid any possible degradation of the active principles in the interval between when the sampleswere taken and when they were evaluated.

In order to determine any possible variability of the samples, at the time they were frozen, duplicates were prepared of each with amounts of active principle that were less than, equal to and greater than the theoretical content. One of each duplicate sample was analyzed by HPLC and the rest were analyzed after having been frozen for one month. There were no variations observed in the composition of either drug.

The interest of this procedure consists in the methodology of the study since, once all the samples were obtained, they were jointly prepared for analysis via HPLC, thus avoiding potential variations in chromatography conditions. The assay was done in triplicate (series nr. 1, 2, and 3).

4. *Analysis instrumentation*
- LKB Bromma 2150 Pump (Pharmacia; Uppsala, Sweden)
- Rheodyne 7125 injector with 20-ml loop
- Kontron 430 HLP detector (Kontron instruments; Milan, Italy)
- Spherisorb 25-mm Ods 15 x 0.46 cm column (Teknokroma; St Cugat, Spain)
- Data acquisition system: Interface for AT-compatible micro-computer Penel-son 900 and related software (Perkin-Elmer; Norwalk, Connecticut, U.S.A.)

5.*Determination of Propofol*
The concentration of propofol in the samples was determined by HPLC using the method of Adam et al. For the determination of Diprivan® in blood[8].

5.1 Material and Methods
5.1.1.Reagents
5.1.1.1. Mobile Phase
- Ultrapure water (purification via mili-Q system [Millipore])
- Acetonitrile for liquid chromatography (Hipersolv)
- Tetrabutylammonium acetate (Merck)
- Trifluoroacetic acid (Merck)

5.1.1.2. Sample Preparation
- Methanol for liquid chromatography (Hipersolv)
- Intralipid " 20% intravenous emulsion (Pharmacia Lot L3)
- Dormicum" (Roche Lot K1)
- Diprivan" (Zeneca Lot K14)
- Distillated water for injectables

5.1.1.3 Development of the Internal Pattern
- Methanol for liquid chromatography (Hipersolv)
- Timol (Panreac)
- Propofol (Zeneca)

5.1.2.Operative Conditions
5.1.2.1 Sample preparation
The samples obtained from the perfusion system were mixed and homogenized by vortex agitation for three minutes. Dilutions of 1/10 ml of the sample were prepared by addition of 1 ml of the internal pattern (timol 0,6 mg/ml), 5 ml of Intralipid" 20% and taken to volume with methanol.

5.1.2.2 Temperature
Perfusion was always done at external temperature of 24±1 °C.

5.1.2.3 Mobile Phase
Acetonitrile-water-tetrabutyl ammonium acetate-trifluoroacetic acid (45:55:3 mM:0.01).

5.1.3 Chromatographic Conditions
Operations were at a flow of 1.0 ml/minute. Sample analysis was performed by reverse-phase HPLC via a UV detector set at 275 nm.

5.1.4 Quantification
Regression lines were drawn with patterns for low, medium and high concentrations in order to calculate the real concentrations of the results obtained in form of absorbances in each chromatogram. Before the assays, a method calibration line was established that confirmed its linearity in a range of concentrations that included those expected for the analyzed samples (see section 7).

6. Determination of Midazolam
Midazolam concentration was determined by a modification of Sautou et al. method[9] for determination of midazolam and two of its metabolites in plasma.

6.1 Material and Methods
6.1.1 REAGENTS
6.1.1.1 Mobile Phase
- Ultrapure water (purification via a mili-Q system [Millipore])
- Phosphate buffer
- Methanol for chromatography (Hipersolv)
- Acetonitrile for liquid chromatography (Hipersolv)
- Tetrahydrofuran for chromatography (Probus)

6.1.1.2 Sample Preparation
- Methanol for liquid chromatography (Hipersolv)
- Intralipid® 205 intravenous emulsion (Pharmacia Iberia Lot L3)*
- Dormicum® (Roche Lot K1)
- Diprivan® (Zeneca Lot K14)
- Water for injectables

6.1.1.3 Development of the Internal Pattern
- Diazepam (Prodesfarma)
- Methanol for liquid chromatography (Hipersolv)

6.1.2 Operative Conditions
6.1.2.1 Sample preparation
Samples were thawed and agitated to homogenize them (3 minutes). Dilutions of 1 ml of the sample, 1 ml of diazepam (internal pattern) 0.6 mg/ml, 1 ml of propofol solution 0,8 mg/ml in methanol and 2.5 ml of Intralipid" 10% and taken to 5ml with methanol were prepared.

6.1.2.2 Temperature
Perfusion was always done at external temperature of 24±1 °C.

6.1.2.3 Mobile Phase
Phosphate buffer-methanol-acetonitrile-tetrahydrofuran (50:25:28:4).

6.1.3 Chromatographic Conditions
Sample analysis was performed by reverse-phase HPLC via a UV detector set at 254 nm. Flow : 1.5 ml/minute.

6.1.4 Quantification
The same method was used as for propofol.

7. Validation of the Method
7.1 Midazolam
7.1.1 Linearity
To determine the method linearity, seven samples of the substance's standart pattern between 0.03mg/ml and 0.21mg/ml, corresponding to concentrations range from 25% to 175% of the theoretical value were prepared. These concentrations were: 0.03, 0.06, 0.09, 0.12, 0.15, 0.18 and 2.1 mg/ml.Each sample was analyzed in triplicate.

From the obtained results, the equation for the correspondng regression line and the r and r2 values were:

A=-4.385301806 r= 0.9996
B= 0.0864325007 r2=0.993

For 19 (n-2) degrees of freedom, the r value obtained had a positive correlation with a probability greater than 99.9%, since the r value for the same levels of significance and degrees of freedom is 0.665<0.9996 (experimental value).

7.1.2 Repeatability
Range of analytical method for determination of midazolam: 0.03-0.21 mg/ml; Expected concentration value : 0.12 mg/ml. For the repeatability study, three different types of samples were used (low, medium and high concentrations) corresponding to concentrations ranging from 50% to 150% of the theoretical value (0.06, 0.12 and 0.18 mg/ml). The analysis of the three samples was done on the same day and each one was analyzed six times. Table 1 shows the obtained results. (Table 1)

Table 1. Midazolam sample analysis for method repeatability study

Concentration(mg/ml)	No. analysed	Mean area±SD	CV(%)
0.06	6	0.6901546857±2.8E-03	0.41
0.12	6	1.416824763±0,017	1.19
0.18	6	2.101190775±0.015	0.71

In the analysis of the finished product according to "Validation of Analytical Methods", a monograph published by A.E.F.I.(Spanish Association of Industry Pharmacists) and wich shall here inafter be referred to as the VMAAEFI, variation coefficients less than 2-3% are considered acceptable for the repeatability assay, and it can therefore be stated that the method has good repeatability.

7.1.3 Reproducibility
Using the three identical concentrations, analyses were run

Table 2. Midazolam sample analysis on four consecutive days for method reproducibility study.

Concentration(mg/ml)	Area				Mean area±SD	VC(%)
	Day 1	Day 2	Day 3	Day 4		
0.06	0.685	0.687	0.689	0.689	0.69025±7.7E-03	1.12
	0.709	0.685	0.689	0.689		
0.12	1.383	1.382	1.386	1.412	1.39175±0.015	1.08
	1.386	1.377	1.418	1.390		
0.18	2.074	2.097	2.088	2.094	2.08875±0.02	0.98
	2.076	2.073	2.133	2.075		

on four consecutive days. Each sample was analyzed twice. Table 2 shows the results obtained. (Table 2)

In according to VMAAEFI, variation coefficients less than 4-5% are considered acceptable and confirm the good reproducibility of the method.
7.2 Propofol
7.2.1 Linearity
To determine linearity of the method used to evaluate the concentration of propofol, samples of 0.2, 0.4, 0.6, 0.8, 1.0, 1.2 and 1.4 mg/ml, corresponding to concentrations ranging from 25% to 175% of the theoretical concentration were prepared.
Each sample was analyzed in triplicate. From the results obtained, the equation for the corresponding regression line and the r and r2 values were:
A = 2.8622 E-3 r = 0.9999
B = 0.8245237619 r2 = 0.9998

For 19 (n-2) degrees of freedom, the r value obtained had a positive correlation with a probability greater than 99.9%, since the r value for the same levels of significance and degrees of freedom is 0.665< 0.9999 (experimental value).

7.2.2 Repeatability
Range of the analytical method for determination of propofol: 0.2-1.4 mg/ml, Expected concentration value : 0.8 mg/ml.
For the repeatability method study, three different types of samples were used corresponding to concentrations ranging from 50% to 150% of the theoretical value (0.4, 0.8 and 1.2 mg/ml). The analysis of the three samples was done on the same day and each one was analyzed six times. Table 4 shows the results. (Table 3)

Table 3. Propofol sample analysis for method repeatability study.

Concentration(mg/ml)	No. analysis	Mean area±SV	CV(%)
0.4	6	0.3334458133±2.41E-3	0.72
0.8	6	0.6660411057±3.67E-3	0.55
1.2	6	0.8344803092±4.28E-3	0.51

In the reproducibility assay acording to VMAAEFI, variation coefficients less than 4-5% confirm the good reproducibility of the method.

Results
In each of the three assays series the drugs concentrations was evaluated, in triplicate, as a function of time. Tables 4 and 5 show the obtained results. (Table 4) (Table 5)

Analysis of experimental results

For each of the three series (each of wich was done intriplicate), a bidirectional variance study was done by Statgraphics Plus program in order to determine if there were statistically significant differences between series, that is, differences in concentrations between the for propofol and midazolam. (Table 6)

Table 4. Midazolam concentration as a function of time.

Time (hours)	Series no.1 (X ± SD)	Series no.2 (X ± SD)	Series no.3 (X ± SD)
t0	0.129±0	0.128±0	0.1286±5.77E-4
t1	0.1283±5.77E-4	0.128±0	0.1286±5.77E-4
t2	0.1271±E-3	0.1263±5.77E-4	0.1273±5.77E-4
t3	0.126±0	0.1253±5.77E-4	0.1263±5.77E-4
t4	0.1256±1.15E-3	0.1253±1.15E-3	0.125±1E-3
t5	0.125±0	0.1243±5.77E-4	0.12483±9.83E-4
t6	0.124±0	0.124±1E-3	0.1236±5.77E-4
t7	0.123±1E-3	0.122E-3	0.123±0
t8	0.12283±7.53E-4	0.1223±5.77E-4	0.122±0
t9	0.1206±5.77E-4	0.1206±E-3	0.1216±5.16E-4
t10	0.120±0	0.1196±5.77E-4	0.120±0
t11	0.1193±1.15E-3	0.11835.77E-4	0.119±0
t12	0.119±0	0.1176±5.77E-4	0.1183±5.77E-4
t12	0.12±0	0.1183±5.77E-4	0.119±1E-3
t13	0.12±0	0.118±0	0.119±1E-3
t14	0.1183±5.77E-4	0.1173±5.77E-4	0.118±0
t15	0.1176±1.15E-3	0.117±0	0.1173±5.77E-4
t16	0.117±0	0.1168±2.66E-4	0.116±5.77E-4
t17	0.1163±5.77E-4	0.116±0	0.116±5.77E-4
t18	0.11563±1.1E-3	0.1146±5.77E-4	0.1153±5.77E-4
t19	0.1146±1.15E-3	0.114±0	0.1143±5.77E-4
t20	0.1136±1.15E-3	0.1133±5.77E-4	0.1136±5.77E-4
t21	0.113±5.77E-4	0.113±0	0.1133±5.77E-4
t22	0.1126±5.77E-4	0.113±0	0.1126±5.77E-4
t23	0.112±0	0.112±0	0.116±5.77E-4
t24	0.1116±5.77E-4	0.1113±1.15E-3	0.1113±5.77E-3
t24	0.112±2E-3	0.116±5.77E-4	0.1116±5.77E-4
t25	0.1126±5.77E-4	0.11131.15E-3	0.1113±5.77E-4
t26	0.111±0	0.1106±5.77E-4	0.1106±5.77E-4
t27	0.1086±3.21E-3	0.109±0	0.109±0
t28	0.109±0	0.1086±5.77E-4	0.1086±5.77E-4
t29	0.1073±1.15E-3	0.1066±1.15E-3	0.1066±1.15E-3
t30	0.1053±1.15E-3	0.106±0	0.106±0
t31	0.105±0	0.1046±5.77E-4	0.1046±1.15E-3
t32	0.1036±1E-3	0.1033±1.15E-3	0.1033±1.15E-3
t33	0.103±1E-3	0.103±0	0.103±0
t34	0.103±0	0.1023±5.77E-4	0.1023±5.77E-4
t35	0.102±0	0.101±0	0.101±0
t36	0.102±0	0.101±1E-3	0.101±1E-3

Table 5. Propofol concentration as a function of time

Time (hours)	Series no.1 (X ± SD)	Series no.2 (X ± SD)	Series no.3 (X ± SD)
t0	0.8203±1.15E-3	0.8063±1.15E-3	0.8146±1.53E-3
t1	0.8106±1.15E-3	0.798±0	0.804±1E-3
t2	0.7946±1.15E-3	0.7916±1.15E-3	0.7943±1.15E-3
t3	0.7906±1.15E-3	0.7856±1.15E-3	0.788±0
t4	0.7853±1.15E-3	0.7793±1.15E-3	0.7826±1.15E-3
t5	0.773±0	0.7693±1.15E-3	0.7763±5.77E-4
t6	0.7695±3.89E-3	0.763±1.15E-3	0.766±2E-3
t7	0.7583±5.77E-4	0.7506±1.15E-3	0.7563±1.15E-3
t8	0.7533±1.15E-3	0.745±1.73E-3	0.748±1E-3
t9	0.7493±1.15E-3	0.7406±5.77E-4	0.743±1.15E-3
t10	0.7466±1.15E-3	0.7376±1.15E-3	0.737±1E-3
t11	0.7406±1.15E-3	0.7293±2.31E-3	0.7356±1.15E-3
t12	0.7366±1.15E-3	0.7243±1.15E-3	0.731±0
t12	0.7436±1.53E-3	0.7763±5.77E-4	0.736±1.15E-3
t13	0.7373±5.77E-4	0.711±5.77E-4	0.725±0
t14	0.7316±1.53E-3	0.694±0	0.7183±5.77E-4
t15	0.7253±1.15E-3	0.686±1E-3	0.712±1E-3
t16	0.7166±1.15E-3	0.6776±1.15E-3	0.6983±5.77E-4
t17	0.7053±5.77E-4	0.6706±5.77E-4	0.6916±5.77E-4
t18	0.6936±1.15E-3	0.6636±1.15E-3	0.6853±5.77E-4
t19	0.687±0	0.6576±5.77E-4	0.6733±5.77E-4
t20	0.6796±2.08E-3	0.651±1E-3	0.667±1E-3
t21	0.6733±1.15E-4	0.646±5.77E-4	0.6516±1.15E-3
t22	0.6656±5.77E-4	0.6406±1.15E-3	0.6446±1.15E-3
t23	0.6576±5.77E-4	0.635±2E-3	0.639±1E-3
t24	0.651±1.73E-3	0.629±0	0.6326±5.77E-4
t24	0.6623±1.15E-3	0.633±1E-3	0.646±1E-3
t25	0.6563±1.15E-3	0.626±1E-3	0.6393±1.15E-3
t26	0.6483±5.77E-4	0.621±1E-3	0.6336±5.77E-4
t27	0.6393±5.77E-4	0.6183±1.15E-3	0.6276±5.77E-4
t28	0.6346±5.77E-4	0.608±0	0.6216±5.77E-3
t29	0.6266±5.77E-4	0.5946±1.15E-3	0.6086±1.15E-3
t30	0.618±0	0.5876±1.15E-3	0.6023±5.77E-4
t31	0.6113±5.77E-4	0.5816±1.15E-3	0.5943±1.15E-3
t32	0.5933±5.77E-4	0.5753±5.77E-3	0.5876±1.15E-4
t33	0.5866±5.77E-4	0.569±0	0.5836±5.77E-4
t34	0.5813±5.77E-4	0.562±0	0.5763±5.77E-4
t35	0.5766±1.15E-3	0.556±5.77E-4	0.5653±2.31E-3
t36	0.572±2E-3	0.5486±1.15E-3	0.553±5.77E-4

ANOVA gives values of $p>0.05$ in all cases, wich allows us to conclude that there are no statistically significant differences among the drug concentration values obtained at the different sampling times in three series. The corresponding mean values can therefore be considered representative of the process.

Obtained results demonstrate that the preparation of this treatment 24 hours in advance is viable, but not 36 hours in advance, owing to the high losses of the original value of drug concentrations in the mixture. Thus, in order to ensure that the perfusion remains within 90% of the original value (drug stability limits considered acceptable), an approximately 10% overdose of each drug is recommended. At any event, it would be necessary to determine in vivo whether or not these differences endure to a significant degree.

Time, more than light or heat, appears to influence the degradation of both drugs. Table 8 summarizes the remaining drugs concentrations as a function of time under the above experimental conditions.

Conclusions

1. Two HPLC methods are updated and validated for the evaluaton of an association of midazolam and propofol in injectable mixtures.

Table 6. Results of variance analysis.

Drug /Time (hours)	Variance	Sum of the squares	D f	F	P
Propofol/0-12	Interseries	0.000432179	2	0,29	0,7521
	Intraseries	0.0270863	2		
	Total	0.275185	2		
Propofol/12-24	Interseries	0.005440897	36	2.65	0,844
	Intraseries	0.3118262	36		
	Total	0.0421497	36		
Propofol/24-36	Interseries	0.00401328	38	2.27	0.1179
	Intraseries	0.000420769	38		
	Total	0.000423692	38		
Midazolam/0-12	Interseries	0.00000292308	2	0,13	0,8828
	Intraseries	0.000420769	2		
	Total	0.000423692	2		
Midazolam/12-24	Interseries	0.00000389744	36	0,27	0,7619
	Intraseries	0.000256	36		
	Total	0.000259897	36		
Midazolam/24-36	Interseries	0.00000389744.	38	0,12	0,8884
	Intraseries	0.000591077	38		
	Total	0.000594974	38		

2. The preparation for 12-hour treatment is viable, given that the loss in concentration of each drug is<10% during this interval. The dose administered is therefore always at least 90% of the initial dose, a limit is customarily accepted in clinical practice.

3. The preparation of mixtures for perfusion after a 12.hour period of refrigeration leads to in vitro concentration losses that approach 10% of the initial dose, even before the start of perfusion. Losses that occur during perfusion must be taken into consideration as well. At the end of the perfusion period (24 hours), the following losses in concentration were detected: midazolam, approximatively 14%;

Table 7. Percent loss under the experimental conditons described.

Experimental conditions	Midazolam	Propofol
t0-12		
Preparation + perfusion	9,1±0	10,2±0,03
t12-24		
Refrigerated 12 h	7,75±0	9,28±0,25
Perfusion	9,3±0	13,71±1,19
Overall	13,95±0	21,84±1,10
t24-36		
Refrigerated 24 h	13,18±0,¿?	20,46±1,11
Perfusion	11,01±2,89	13,75±0,58
Overall	22,74±2,5	31,39±1,00

and propofol, 22%. An initial overdose could be proposed as a possible palliative measure (5% midazolam and 10% propofol).

*Since Diprivan® uses as a vehicle for propofol an injectable lipid emulsion that meets the requirements for intravenous injection, the blanks used for the samples preparation are dilutions of the same in a fixed amount of a vehicle of similar characteristics: Intralipid® 20%.

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