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DETERMINATION OF MIDAZOLAM (VERSED®) AND ITS METABOLITES IN PLASMA BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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SUMMARY

A rapid, sensitive and selective high-performance liquid chromatographic (HPLC) assay was developed for the determination of midazolam, 8-chloro-6-(2-fluorophenyl)-1-methyl-4H-imidazo[1,5-a][1,4]-benzodiazepine (I), and its four metabolites in plasma. The assay involves extraction into diethyl ether—methylene chloride (7:3) from plasma buffered to pH 9 and subsequent analysis by reversed-phase HPLC with ultraviolet detection at 254 nm. The overall recovery of I from dog plasma was 94.5 ± 7.1% and > 89.0% for its metabolites. The sensitivity limit of the assay was 50 ng/ml of plasma for all compounds. The HPLC assay was used to determine plasma concentrations of I and its metabolites from selected samples taken from an oral toxicity study in the dog.

INTRODUCTION

The compound, midazolam, 8-chloro-6-(2-fluorophenyl)-1-methyl-4H-imidazo[1,5-a][1,4]-benzodiazepine, Versed® (I) (Fig. 1), is under clinical development as a parenterally administered short-acting anesthesia-inducing agent [1] and orally as a hypnotic [2].

Sensitive and specific assays have been reported for the quantitation of midazolam (I) and its major 1-hydroxymethyl metabolite, II (Fig. 1) in blood, plasma and urine in man. These methods include gas chromatography with electron-capture [3—8] and nitrogen-selective flame-ionization [9, 10] detection, high-performance liquid chromatography (HPLC) [10—12], gas chromatography—negative-ion chemical-ionization mass spectrometry [13], midazolam per se by radioimmunoassay [14], and by polarographic detection [3].

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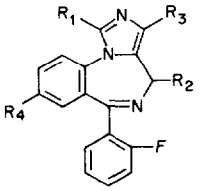

COMPOUND	R_1	R_2	R_3	R_4
Midazolam [I]	$-CH_3$	-H	-H	-Cl
1-Hydroxymethylmidazolam [II]	$-CH_2OH$	-H	-H	-Cl
4-Hydroxymidazolam [III]	$-CH_3$	-OH	-H	-Cl
4-Hydroxy-1-hydroxymethyl-midazolam [IV]	$-CH_2OH$	-OH	-H	-Cl
1-Desmethylmidazolam [V]	-H	-H	-H	-Cl
Internal standard [VI]	-H	-H	$-CO_2C_2H_5$	-I

Fig. 1. Chemical structures for the compounds referred to in the text.

In addition to the 1-hydroxymethyl metabolite (II), other minor biotransformation products have been reported in man and in the rat [6, 12, 15, 16] (Fig. 1). A sensitive and specific HPLC assay with automated injection was developed in order to simultaneously quantitate midazolam and its metabolites.

The method presented herein determines midazolam (I), the 1-hydroxymethyl metabolite (II), the 4-hydroxy metabolite (III), the 4-hydroxy-1-hydroxymethyl metabolite (IV), and the desmethyl metabolite (V) by reversed-phase HPLC using their ultraviolet (UV) absorbance at 254 nm for quantitation. The iodo analogue, VI (Fig. 1), is used as the internal standard.

The ease of operation and selectivity of the method in quantitating midazolam and its four metabolites make it especially useful in the analysis of plasma samples taken during toxicology studies.

The HPLC assay was used to determine plasma concentrations of I and II from selected samples taken from an oral toxicity study in the dog.

EXPERIMENTAL

High-performance liquid chromatography of compounds I—V in plasma

Columns. A prepacked μBondapak C_{18} 10-μm (30 cm × 3.9 mm I.D.) (Waters Assoc., Milford, MA, U.S.A.) column or an IBM C_{18} 5-μm (25 cm × 4.5 mm I.D.) (IBM Instruments, Danbury, CT, U.S.A.) column was used.

Instrumental parameters. The HPLC system consisted of a Model M6000A reciprocating piston pump, a Waters intelligent sample processor (WISP™) Model 710B, and a Model 440 fixed-wavelength UV detector (Waters Assoc.). The isocratic mobile phase used was a mixture of methanol—acetonitrile—0.01 *M* potassium phosphate buffer (pH 7.4)—tetrahydrofuran (30:28:40:2) for the Waters column and 29:28:41:2 for the IBM column, at a pressure of ca. 10.5 MPa and a constant flow-rate of 1.3 ml/min. The UV detector was operated at

TABLE I

RETENTION TIMES (t_R) AND CAPACITY FACTORS (k') OF COMPOUNDS I–VI ON WATERS μBONDAPAK C_{18} AND IBM C_{18} COLUMNS

Compound	Waters μBondapak C_{18}		IBM C_{18}	
	t_R (min)	k'	t_R (min)	k'
I	6.2	1.67	8.1	2.59
II	4.6	1.00	5.9	1.62
III	4.3	0.83	5.4	1.38
IV	3.5	0.50	4.2	0.84
V	5.3	1.25	6.8	2.03
VI	7.1	2.02	11.7	4.17

254 nm at a sensitivity of 0.01 a.u.f.s. and the chart speed on the 10-mV recorder (Model 7132A, Hewlett-Packard, Palo Alto, CA, U.S.A.) was 1.27 cm/min. The WISP auto-injector was programmed to run for 12–15 min per sample using methanol as the rinse solvent. Under these conditions 80 ng of I, 60 ng of II or V, 40 ng of III or IV and 50 ng of VI injected gave nearly full scale pen responses. The retention times (t_R) in minutes and capacity factors (k') of I through VI depending on the column used are listed in Table I. The minimum detectable amount of each compound was 5.0 ng injected, equivalent to 50.0 ng/ml of plasma.

Reagents

All reagents were of analytical-reagent grade (> 99% purity) and include acetonitrile, methanol, methylene chloride, tetrahydrofuran (Burdick & Jackson Labs., Muskegon, MI, U.S.A.) and anhydrous diethyl ether (Mallinckrodt, Paris, KY, U.S.A.). Potassium phosphate buffer (1.0 *M*, pH 9) is prepared by mixing 100 ml of 1.0 *M* potassium dihydrogen phosphate with 900 ml of 1.0 *M* dipotassium hydrogen phosphate; potassium phosphate buffer (1.0 *M*, pH 7.4) is prepared by mixing 390 ml of 1.0 *M* potassium dihydrogen phosphate with 610 ml of 1.0 *M* dipotassium hydrogen phosphate. The 0.01 *M* pH 7.4 buffer is prepared by diluting 10.0 ml of the 1.0 *M* pH 7.4 buffer to 1 l with distilled deionized water.

Analytical standards

Compound I, 8-chloro-6-(2-fluorophenyl)-1-methyl-4H-imidazo[1,5-a][1,4]-benzodiazepine ($C_{18}H_{13}Cl\ FN_3$, M.W. 325.8, m.p. 152–154°C); compound II, 8-chloro-6-(2-fluorophenyl)-1-hydroxymethyl-4H-imidazo[1,5-a][1,4]-benzodiazepine ($C_{18}H_{13}ClFN_3O$, M.W. 341.8, m.p. 258–260°C); compound III, 8-chloro-6-(2-fluorophenyl)-4-hydroxy-1-methyl-4H-imidazo[1,5-a][1,4]-benzodiazepine ($C_{18}H_{13}ClFN_3O$, M.W. 341.8, m.p. 185–186°C); compound IV, 8-chloro-6-(2-fluorophenyl)-4-hydroxy-1-hydroxymethyl-4H-imidazo[1,5-a]-[1,4]-benzodiazepine ($C_{18}H_{13}ClFN_3O_2$, M.W. 357.8, m.p. 238–240°C); compound V, 8-chloro-6-(2-fluorophenyl)-4H-imidazo[1,5-a][1,4]-benzodiazepine ($C_{17}H_{11}ClFN_3$, M.W. 311.8, m.p. 150–151°C); and compound VI, 6-(2-fluorophenyl)-8-iodo-4H-imidazo[1,5-a][1,4]-benzodiazepine-3-carbox-

ylic acid ethylester ($C_{20}H_{15}FIN_3O_2$, M.W. 475.3, m.p. 200—202°C), of pharmaceutical grade purity (> 99%), are used as analytical standards.

Preparation of analytical standards

Prepare stock solutions of compounds I—VI in separate 10-ml volumetric flasks by dissolving 10 mg of each compound into 2 ml of methanol. Sonicate if necessary for 5—10 min for complete solubilization and dilute to volume with methanol. These stock solutions (containing 1.0 mg/ml) are used to prepare the mixed standard solutions 1—9 by suitable dilutions in methanol to contain the amounts as indicated in Table II. Aliquots (100 μl) of solutions 1—8 are added to separate 1.0-ml specimens of control plasma and processed along with the samples to establish a processed (recovered) standard calibration curve for the direct quantitation of unknowns. Aliquots (10 μl) of solutions 1—8 are injected as the external standard calibration curve to establish the linearity and performance of the HPLC system.

Calibration of compounds I—V by HPLC

Calibration (external standard) curves of the peak-height ratio of I—V to VI versus concentration of compound injected are constructed. Fresh calibration curves of the external standards and of the processed (recovered) standards are prepared for each day of analysis to establish the linearity and reproducibility of the HPLC system.

Analysis of plasma

The flow diagram of the extraction procedure is shown in Fig. 2. Into a 15-ml conical centrifuge tube (PTFE No. 13, stoppered) add 1 ml of plasma, 100 μl of standard solution 9 (equivalent to 500 ng of VI, the internal standard), 2 ml of 1 *M* pH 9.0 potassium phosphate buffer and vortex. Extract with 8.0 ml of diethyl ether—methylene chloride (7:3) by shaking for 15 min on a reciprocating shaker (Eberbach, Ann Arbor, MI, U.S.A.) at 60—80 strokes/min.

Along with the samples, process nine 1.0-ml specimens of control plasma, one to be used as a control blank to which 100 μl of methanol are added and

TABLE II

PREPARATION OF THE MIXED STANDARD SOLUTIONS 1—9

Standard solution No.	Concentration (ng per 100 μl)					
	I	II	III	IV	V	VI
1	50	50	50	50	50	500
2	100	100	100	100	100	500
3	200	200	200	200	200	500
4	400	300	300	300	300	500
5	800	600	400	400	600	500
6	1000	1000	1000	1000	1000	500
7	2000	2000	2000	2000	2000	500
8	4000	4000	4000	4000	4000	500
9	—	—	—	—	—	500

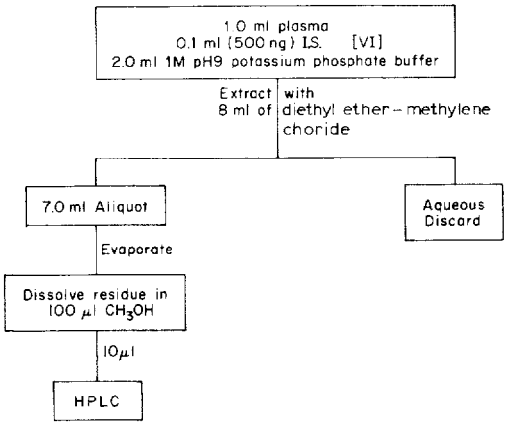

Fig. 2. Flow diagram for the analysis of compounds I, II, III, IV and V from plasma.

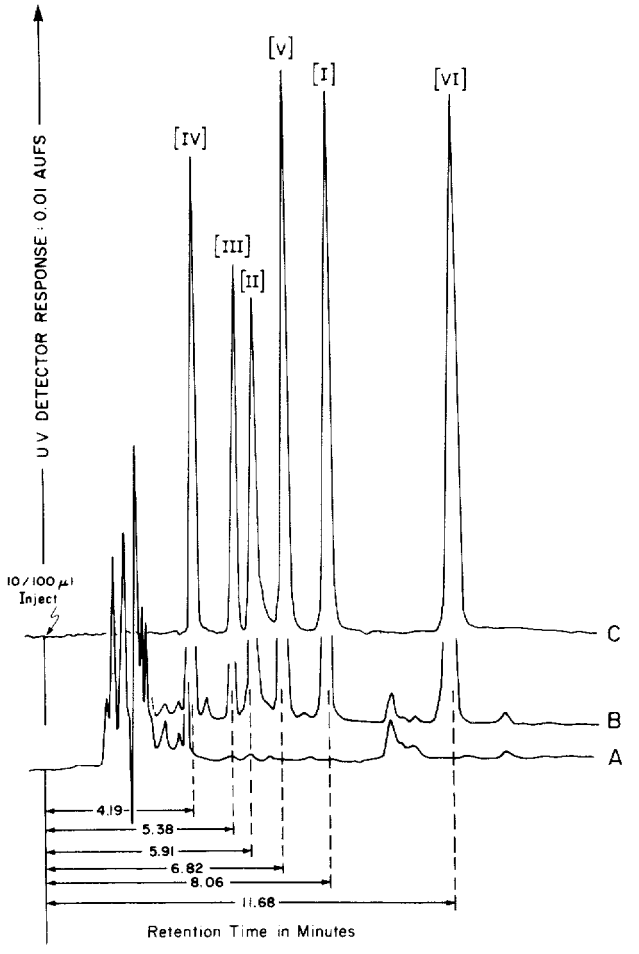

Fig. 3. Chromatograms of the HPLC analysis of extracts of (A) control dog plasma, (B) authentic standards recovered from control dog plasma, and (C) authentic standards of compounds I–VI.

eight to be used for the preparation of the processed (recovered) standards to which 100 μl of solutions 1—8 are added. Centrifuge the samples in a refrigerated centrifuge (Model PR-J, Rotor No. 253, Damon/IEC, Needham, MA, U.S.A.) at 5°C for 5—10 min at 2100 rpm (1500 *g*). Transfer a 7.0-ml aliquot of the upper organic layer into a second 15-ml centrifuge tube and evaporate to dryness at 40—45°C in an N-EVAP evaporator (Organomation Assoc., Worchester, MA, U.S.A.) under a stream of clean, dry nitrogen. Dissolve the residue in 100 μl of methanol and transfer the contents into a low-volume glass insert (Waters Part No. 72704) fitted into a standard 4-ml glass vial (Waters Part No. 72710) and sealed with a self-seal septum (Waters Part No. 73010). Program the auto-injector (WISP 710B) to inject 10 μl for HPLC analysis. Typical chromatograms of plasma extracts are shown in Fig. 3.

Calculations and assay validation

The concentrations of I and, if present, of any of the metabolites II—V in the unknowns are determined by interpolation from a least-squares regression equation (power equation: $y = mx^b$) of the calibration data (processed by a Hewlett-Packard Model 3357B Laboratory Automation System) of the recovered standards processed along with the unknowns using peak-height ratios (peak height of compound I, II, III, IV or V to peak height of the internal standard, VI) versus concentration of I, II, III, IV or V per ml of plasma. Typical calibration curve data for I—V are linear from 50.0 to 4000 ng/ml for each compound (Table III). Intra- and inter-assay validation data over the same concentration range for I—V are summarized in Tables IV—VIII.

The determination of percentage recovery is calculated (with each analytical experiment) by comparing the absolute response (peak height) of the processed (recovered) standards to the absolute response (peak height) of the external standards.

RESULTS AND DISCUSSION

A rapid, sensitive and selective HPLC assay was developed for the simultaneous determination of midazolam, I (Versed®) and its four metabolites from 1.0 ml of plasma using UV detection at 254 nm for quantitation. This method enabled the rapid and accurate quantitation of I and the metabolites

TABLE III

TYPICAL CALIBRATION CURVE DATA FOR I—V FROM THE PROCESSED (RECOVERED) STANDARDS USING A LEAST-SQUARES REGRESSION (POWER) EQUATION ($y = mx^b$)

Compound	*m*	*b*	Correlation coefficient (*r*)	Average percentage deviation from the line
I	1.57	1.02	0.999	2.5
II	1.30	1.08	0.999	2.8
III	2.11	0.999	0.999	4.3
IV	2.60	0.983	0.999	3.8
V	2.16	1.02	0.999	3.2

TABLE IV

STATISTICAL VALIDATION OF THE HPLC ASSAY FOR COMPOUND I

Concentration added (μg/ml)	Concentration found (μg/ml)	Coefficient of variation (%)
Intra-assay variability (n = 3)		
0.050	0.054 ± 0.001	1.9
0.100	0.098 ± 0.003	3.1
0.200	0.206 ± 0.013	6.5
0.400	0.409 ± 0.002	0.5
0.800	0.798 ± 0.036	4.5
1.00	1.07 ± 0.05	4.7
2.00	1.94 ± 0.14	7.2
4.00	4.20 ± 0.33	7.9
Average		4.5
Inter-assay variability (n = 5)		
0.050	0.052 ± 0.003	5.8
0.100	0.096 ± 0.005	5.2
0.200	0.199 ± 0.016	8.0
0.400	0.401 ± 0.016	4.0
0.800	0.804 ± 0.067	8.3
1.00	0.982 ± 0.125	12.7
2.00	1.93 ± 0.12	6.2
4.00	4.14 ± 0.28	6.8
Average		7.1

TABLE V

STATISTICAL VALIDATION OF THE HPLC ASSAY FOR COMPOUND II

Concentration added (μg/ml)	Concentration found (μg/ml)	Coefficient of variation (%)
Intra-assay variability (n = 3)		
0.050	0.049 ± 0.002	4.2
0.100	0.104 ± 0.006	5.8
0.200	0.210 ± 0.005	2.4
0.300	0.301 ± 0.007	2.3
0.600	0.611 ± 0.026	4.3
1.00	1.15 ± 0.09	7.8
2.00	1.94 ± 0.15	7.7
4.00	4.30 ± 0.57	13.3
Average		6.0
Inter-assay variability (n = 5)		
0.050	0.051 ± 0.005	9.8
0.100	0.099 ± 0.008	8.1
0.200	0.203 ± 0.014	6.9
0.300	0.290 ± 0.020	6.9
0.600	0.598 ± 0.051	8.5
1.00	1.05 ± 0.16	15.2
2.00	1.90 ± 0.14	7.4
4.00	4.15 ± 0.48	11.6
Average		9.3

TABLE VI

STATISTICAL VALIDATION OF THE HPLC ASSAY FOR COMPOUND III

Concentration added (μg/ml)	Concentration found (μg/ml)	Coefficient of variation (%)
Intra-assay variability (n = 3)		
0.050	0.051 ± 0.001	2.0
0.100	0.102 ± 0.004	3.9
0.200	0.198 ± 0.005	2.5
0.300	0.302 ± 0.004	1.3
0.400	0.408 ± 0.020	4.9
1.00	1.12 ± 0.10	8.9
2.00	1.91 ± 0.18	9.4
4.00	4.30 ± 0.55	12.8
Average		5.7
Inter-assay variability (n = 5)		
0.050	0.052 ± 0.003	5.8
0.100	0.099 ± 0.008	8.1
0.200	0.192 ± 0.017	8.9
0.300	0.297 ± 0.018	6.1
0.400	0.409 ± 0.049	12.0
1.00	1.04 ± 0.18	17.3
2.00	1.90 ± 0.17	8.9
4.00	4.17 ± 0.47	11.3
Average		9.8

TABLE VII

STATISTICAL VALIDATION OF THE HPLC ASSAY FOR COMPOUND IV

Concentration added (μg/ml)	Concentration found (μg/ml)	Coefficient of variation (%)
Intra-assay variability (n = 3)		
0.050	0.059 ± 0.009	15.3
0.100	0.102 ± 0.005	4.9
0.200	0.212 ± 0.007	3.3
0.300	0.307 ± 0.002	0.7
0.400	0.418 ± 0.020	4.8
1.00	1.13 ± 0.11	9.7
2.00	1.94 ± 0.17	8.8
4.00	4.22 ± 0.60	14.2
Average		7.7
Inter-assay variability (n = 5)		
0.050	0.054 ± 0.005	9.3
0.100	0.098 ± 0.011	11.2
0.200	0.206 ± 0.024	11.7
0.300	0.297 ± 0.033	11.1
0.400	0.411 ± 0.057	13.9
1.00	1.04 ± 0.19	18.3
2.00	1.92 ± 0.17	8.9
4.00	4.06 ± 0.50	12.3
Average		12.1

TABLE VIII

STATISTICAL VALIDATION OF THE HPLC ASSAY FOR COMPOUND V

Concentration added (μg/ml)	Concentration found (μg/ml)	Coefficient of variation (%)
Intra-assay variability (n = 3)		
0.050	0.053 ± 0.003	5.7
0.100	0.100 ± 0.001	1.0
0.200	0.203 ± 0.003	1.5
0.300	0.308 ± 0.003	1.0
0.600	0.599 ± 0.030	5.0
1.00	1.07 ± 0.06	5.6
2.00	1.88 ± 0.15	8.0
4.00	4.28 ± 0.50	11.7
Average		4.9
Inter-assay variability (n = 5)		
0.050	0.052 ± 0.003	5.8
0.100	0.098 ± 0.004	4.1
0.200	0.196 ± 0.014	7.1
0.300	0.302 ± 0.012	4.0
0.600	0.601 ± 0.046	7.7
1.00	1.03 ± 0.10	9.7
2.00	1.87 ± 0.12	6.4
4.00	4.25 ± 0.35	8.2
Average		6.6

with the high sample throughput required for preclinical pharmacokinetic and toxicological studies. Compounds I—V exhibited comparable UV absorption at 254 nm. The Waters Model 440 absorbance detector used in conjunction with a 254-nm wavelength kit and a medium-pressure mercury lamp allowed for quantitation of I—VI in the ng/ml concentration range. The selectivity of the assay was achieved by baseline resolution of all six compounds. Several μBondapak C_{18}, 10-μm (30 cm × 3.9 mm I.D.) columns as well as IBM C_{18}, 5-μm (25 × 4.5 mm I.D.) columns were used during this project. Minor modification of the mobile phase was necessary due to variability between the columns. Compound VI, an iodo analogue, was chosen as the internal standard in the assay, due to its similar extraction and chromatographic behavior to compounds I—V. Compound VI has not been identified as a metabolite of compound I.

Percentage recovery, sensitivity limits and specificity

The overall recoveries of compounds I, II, III, IV and V are 94.5 ± 7.1%, 89.7 ± 8.1%, 92.2 ± 6.7%, 89.3 ± 5.7% and 95.2 ± 7.3% from dog plasma, respectively. The sensitivity limit of the assay was 50.0 ng of each compound per ml of dog plasma, using UV detection at 254 nm. The selectivity of the assay has not been demonstrated against possible co-administered drugs.

Application of the method to biological specimens

The HPLC method was applied to determine the plasma concentration—

TABLE IX

MAXIMUM PLASMA CONCENTRATIONS OF I AND II AT 2 h ON DAYS 1 AND 16 FOLLOWING THE ORAL ADMINISTRATION OF 45 mg/kg I PER DAY TO DOGS

Day	Dog No.	Sex	Concentration at 2 h (μg/ml)	
			I	II
1	D1	F	0.120	0.550
	D4	F	NM*	0.095
	D7	F	0.176	0.104
1	D10	M	0.180	1.550
	D13	M	0.525	2.060
	D18	M	0.176	0.650
16	D1	F	NM	0.110
	D4	F	NM	0.068
	D7	F	NM	0.079
16	D10	M	NM	0.100
	D13	M	NM	0.079
	D18	M	NM	0.185

*NM = Non-measurable (< 0.050 μg/ml).

time profile of compound I and its metabolites from selected samples taken from an oral toxicity study in the dog. Following the oral administration of 45 mg/kg per day of I maximum plasma concentrations of compound I were determined at 2 h on day 1. Maximum plasma concentrations of compound II were measured at 2 h on days 1 and 16 (Table IX). Compounds III, IV and V were non-measurable at any time point.

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