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Simple high-performance liquid chromatographic method to analyse megazol in human and rat plasma

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Abstract

A simple and sensitive high-performance liquid chromatographic method has been developed to measure megazol in human plasma. The method was optimized and validated according to the Washington Concensus Conference on the Validation of Analytical Methods (V.P. Shah et al., Eur. J. Drug Metab. Pharmacokinet., 15 (1991) 249). The criteria of complete validation were specificity, linearity, precision, analytical recovery, dilution and stability. It involved extraction of the plasma with dichloromethane, followed by reversed-phase high-performance liquid chromatography using a Kromasil^{R} C_8 column and UV detection at 360 nm. The retention times of the internal standard (tinidazol) and megazol were 6.10 and 9.60 min, respectively. The standard curve was linear from 2 ng ml^{-1} (limit of quantification) to 2000 ng ml^{-1}. The coefficients of variation for all the criteria of validation were less than 6%; 85 to 92% extraction efficiencies were obtained. Megazol was stable during the storage period (one month at −20°C) in plasma and for two months at 25°C in standard solution. The method was tested by measuring the plasma concentration following oral administration to rat and was shown to be suitable for pharmacokinetic studies. © 1997 Elsevier Science B.V.

Keywords: Megazol

1. Introduction

Megazol is a 5-nitroimidazole thiadiazole (Fig. 1) that was first synthesised in 1968 by Berkelhammer and Asato [1], who screened it for its efficacy against protozoal infections in mice. Recently, Bouteille et al. [2] studied megazol activity against *Trypanosoma brucei brucei* infections in mice. In vitro, megazol exhibited a trypanocidal effect with an MEC_{100} (100% minimal effective concentration) equal to 0.1 μg ml^{-1}, which is ten-times more effective than that of Suramin (MEC_{100}=1 μg ml^{-1}). In vivo, a single 20 mg kg^{-1} injection of megazol cured mice of acute trypanosomal infection. For subacute infection with

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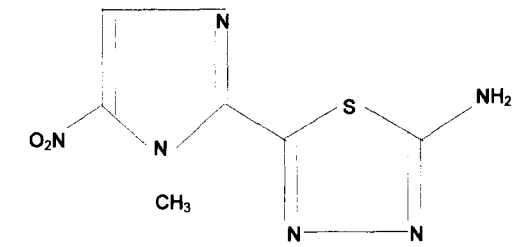
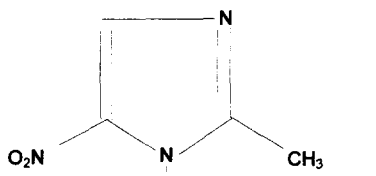


MEGAZOL



CH_2-CH_2-SO_2-CH_2-CH_3

TINIDAZOL (I.S.)

Fig. 1. Structures of megazol and tinidazol.

central nervous system involvement, megazol or Suramin administered alone did not show any effect on the progression of the disease. In contrast, their combined use proved to be extremely effective. It is not known if the trypanocidal effect is due to the parent compound or to one or more of its metabolites. Determination of megazol in biological fluids would clarify whether megazol crosses the blood–brain barrier or not, and would allow pharmacokinetic studies to be carried out in vivo.

The purpose of this paper is to describe a complete optimised high-performance liquid chromatographic (HPLC) method and its full validation according to the Washington Conference Consensus on the Validation of Analytical Methods [3]. The quantification of megazol is validated in human plasma and has been applied to rat plasma.

2. Experimental

2.1. Chemicals

Megazol, 2-amino-5-[1-methyl-5-nitro-2-imidazolyl]1,3,4 thiadiazole was synthesised by G. Chauvière and tinidazol, used as the internal standard, was purchased from Sigma, France (ref. 32H0386).

2.2. Reagents

Chemicals were of analytical grade and human plasma was obtained from Institute Jacques Boy, France.

2.3. Extraction procedure

To 1 ml of plasma, 50 μl of the internal standard solution (10 μg ml^{-1} in mobile phase) and 50 μl of 1 *M* sodium hydroxide were added in a 15-ml conical polypropylene tube with 7 ml of dichloromethane. The samples were mixed on a rotatory agitator for 20 min, then centrifuged at 1636 *g* for 10 min.

After centrifugation, the organic phase (5 ml) was transferred to glass tubes and evaporated to dryness at 45°C under a stream of nitrogen. The dried residues were redissolved by vortex agitation in the mobile phase (200 μl) and 50 μl of the extract was injected onto the HPLC column.

2.4. Chromatographic system

The following modular HPLC system was used: a Model SP-8810 precision isocratic pump at 170 bar (Spectra Physics, Darmstadt, Germany), a Model Spectra chrom 100 UV monitor (Spectra Physics) fitted with a Model SP-8875 automatic sampler (Spectra Physics) and equipped with a 50-μl loop. Data processing and calculations were carried out using a Chrom jet integrator (Spectra Physics) with the help of PCe software WINner/286 (Spectra Physics).

The Kromasil^{R} column (250×4 mm I.D, 10 μm particle size) was packed with C_8 (Bischoff Chromatography, Leonberg, Germany). The mobile phase consisted of 0.068 *M* phosphate buffer, pH 3, containing methanol and acetonitrile (65:20:15, v/v). The flow-rate was set at 0.7 ml min^{-1} and UV detection was at 360 nm. The system was used at room temperature.

2.5. Validation criteria

2.5.1. Specificity

This criteria was validated on analysing human samples from six different subjects.

2.5.2. Analytical recovery

Analytical recovery was determined by comparing the peak areas of extracts to that obtained on direct injection of the same amount of megazol in mobile phase solution. The assay was determined at three concentrations, each of which was analysed in triplicate.

2.5.3. Calibration curves

Working standards were prepared in drug-free plasma (900 μl) from the stock standard (100 μl) to yield concentrations of 2, 5, 10, 50, 500, 1000, 1600 and 2000 ng ml^{-1} (free-base) in plasma. Nine reference samples for calibration were included in the daily series of analysis. The calibration factors were calculated according to least-squares linear regression, weighted by $1/C$.

2.5.4. Intra- and inter-day variability

These assays were determined by HPLC analysis of six quality control (QC) samples for each concentration (5, 110 and 1400 ng ml^{-1}) during the same day (repeatability) and of two samples for each concentration on five consecutive days (reproducibility).

2.5.5. Dilution

The influence of dilution was studied on a blank plasma sample spiked to obtain a concentration that was two-fold higher than the maximal concentration of the calibration curve. It was then diluted to one third with the same plasma.

2.5.6. Stability

The stability of megazol in plasma was evaluated for one month at −20°C, and for three freeze–thaw cycles. The validation criteria were calculated using commonly accepted statistical procedures. The precision and accuracy of each QC value should not have exceeded a deviation of 15%, except for the QC samples near the limit of quantification (LOQ), where 20% was acceptable.

2.5.7. Pharmacokinetics in rat

Megazol concentrations in rat plasma were obtained after a single oral dose of 10 mg kg^{-1} over 24 h.

3. Results and discussion

3.1. Specificity

Fig. 2 illustrates representative chromatograms of a blank plasma and plasma spiked with 10 ng ml^{-1} of megazol and with 500 ng of tinidazol. The two peaks were well separated, with retention times of 6.10 and 9.60 min for internal standard and megazol, respectively, using a mobile phase composed of phosphate buffer–methanol–acetonitrile. No peaks from endogenous compounds that interfered with megazol and tinidazol were observed with the six tested plasma samples.

3.2. Extraction and recovery

Since the evaporation time for a dichloromethane extract was significantly lower (20 min) and gave a very clean extract, this solvent was chosen. The analytical recoveries of megazol at 5, 110 and 1400 ng ml^{-1} were 90.8, 92.2 and 87.5%, respectively ($n=3$) for each concentration level. The recovery of internal standard was also good (93%) at 2500 ng ml^{-1}.

3.3. Linearity and sensitivity

Excellent linearity was observed between the peak area ratios in plasma (megazol/tinidazol) and the theoretical concentrations ($r^2=0.999$). The LOQ was 2 ng ml^{-1} in plasma. The calibration curve data obtained throughout the validation study are summa-

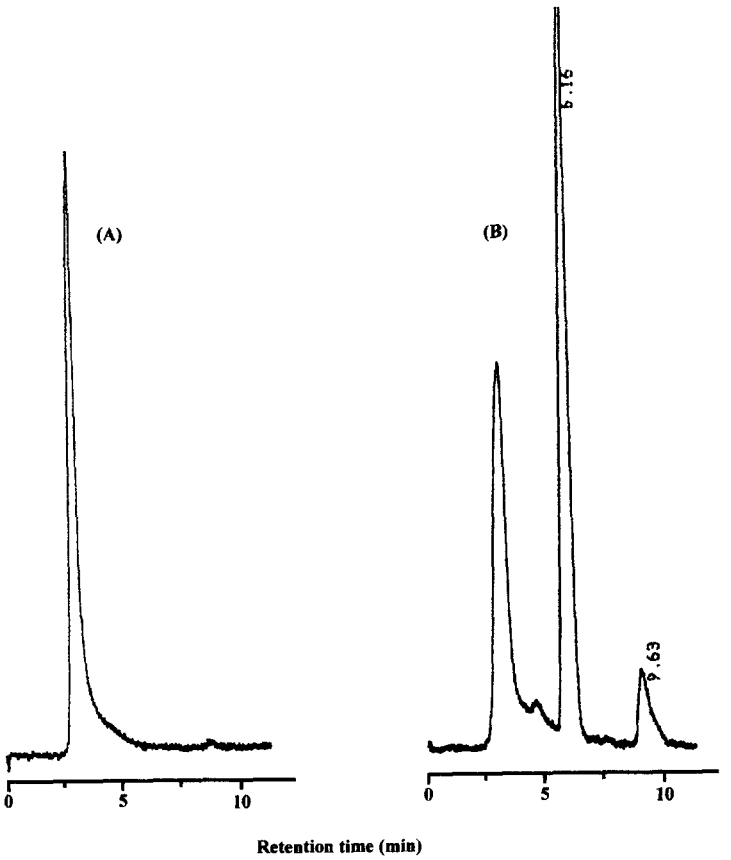


Fig. 2. Chromatograms of plasma extracts. (A) Blank human plasma, (B) human plasma spiked with 10 ng/ml of megazol and 500 ng of tinidazol.

rised in Table 1. The day-to-day slope variability of the calibration curves was 0.57% ($n=5$).

3.4. Influence of dilution

The precision and accuracy of six replicates of diluted plasma were 2.8 and 99.4%, respectively.

3.5. Intra- and inter-day validation

The within- and between-day variations of QC samples are given in Table 2. For each level, the repeatability and reproducibility criteria were clearly satisfactory. The accuracy for both assays (intra- and inter-day) was greater than 90% for all tested concentrations.

3.6. Stability

After four weeks of storage at −20°C, QC samples were shown to be stable at three concentrations (Table 3). No significant degradation of megazol was observed after three freeze–thaw cycles: 101 vs. 102 ng ml^{-1} and 1304 vs. 1332 ng ml^{-1}. The standard solution of 50 ng ml^{-1} that was stored at room temperature was tested six times from the time of its preparation up to 25 days and the results indicate that the solution of megazol was stable. Also, the dry extract was shown to be stable at 4°C for 24 h.

3.7. Application

Fig. 3 illustrates the plasma concentrations measured over 24 h after a single oral dose of 10 mg

Table 1
Calibration curve data (inter-assay precision)

	C (ng/ml)							
	2	5	10	50	500	1000	1600	2000
Day 1	1.971	5.216	8.972	54.632	494.397	984.631	1594.293	2022.888
Day 2	2.225	5.084	9.466	47.091	482.019	1011.481	1617.539	1992.096
Day 3	1.868	4.818	10.737	53.162	482.231	984.086	1613.285	2016.813
Day 4	1.996	5.302	9.775	48.602	487.544	1017.935	1608.090	1987.757
Day 5	2.155	4.496	9.625	53.353	497.733	992.025	1620.301	1987.313
Mean	2.04	4.98	9.71	51.37	488.8	998.0	1610.7	2001.4
S.D.	0.14	0.33	0.65	3.31	7.10	15.71	10.27	17.11
CV %	7.07	6.59	6.65	6.44	1.45	1.57	0.64	0.85
Accuracy %	97.84	99.66	97.15	97.26	97.76	99.8	99.33	99.93

Table 2
Analysis precision of quality control samples

Inter-day precision				Intra-day precision			
	QC1	QC2	QC3		QC1	QC2	QC3
	5	110	1400		5	110	1400
	5.5	119	1410.4		5	112.1	1295.5
	5.5	126.2	1440.2		4.7	119.8	1352.5
	4.1	112.1	1371.9		4.1	109	1371.9
	4.7	119.8	1366.6		5.2	97.7	1366.6
	3.2	118.8	1396.1		5.3	109.8	1396.5
	4.2	119	1370.7		5.1	102.5	1302.8
	3.9	128.6	1396				
	4.9	111.3	1420.4	Mean	4.90	108.5	1347.6
	4.6	120.5	1390.5	S.D.	0.40	7.70	40.2
	4.3	114.6	1362.8	C.V. (%)	8.90	7.10	3.00
				Accuracy (%)	98.00	98.60	96.30
Mean	4.50	119.00	1392.60				
S.D.	0.70	5.50	25.50				
C.V. (%)	16.00	4.60	1.80				
Accuracy (%)	89.60	91.80	99.50				

Table 3
Stability of megazol in human plasma at −20°C

	QC 1	QC 2	QC 3
	4.600	121.7	1365.80
	4.000	107.5	1445.40
	3.500	120.3	1411.2
	3.900	110.9	1442.8
	3.800	116	1297.8
	3.000	118.8	1405.30
Mean	3.800	115.9	1399.70
S.D.	0.500	5.6	58.70
C.V. (%)	14.20	4.80	4.20
Accuracy (%)	75.90	94.70	99.90

kg^{-1} megazol in rats. Each value corresponds to the mean of three samples. This figure shows that the technique is suitable for pharmacokinetic studies of megazol.

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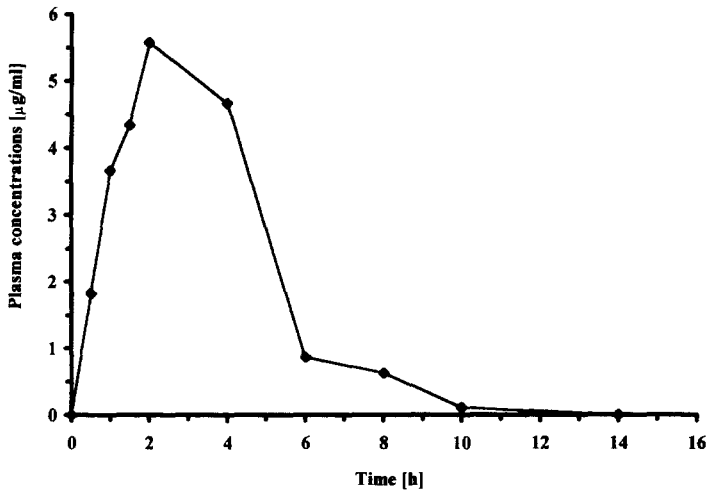

Fig. 3. Plasma concentrations versus time profile of megazol.

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