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Sensitive high-performance liquid chromatographic determination of arbidol, a new antiviral compound, in human plasma[1]

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Abstract

A highly sensitive and selective HPLC method was developed and validated for the determination of arbidol in human plasma. The method involves the liquid–liquid extraction of drug and internal standard from plasma with *tert.*-butyl methyl ether followed by evaporation and reconstitution in mobile phase. UV detection was done at 315 nm. The limit of quantification for arbidol in plasma was 0.005 μg/ml. Linearity in plasma was proven over the whole calibration range (10.2–0.005 μg/ml). The method was validated according to GLP guidelines and its suitability was demonstrated by analysis of samples from a pharmacokinetic study.

Keywords: Arbidol

1. Introduction

Arbidol, ethyl-6-bromo-4-[(dimethylamino)-methyl]-5-hydroxy-1-methyl-2-[(phenylthio)methyl]-indole-3-carboxylate hydrochloride monohydrate (Fig. 1), is an antiviral active chemical entity. It has been in use in Russia for several years for the treatment of influenza. The compound exerts its effect by activation upon phagocytic activity of the macrophages, and also stimulates some forms of cellular and humoral immunity. Arbidol inhibits viruses of influenza type A and type B and has the capacity to induce serum interferon. Metabolic and pharmacokinetic studies in animals have shown that orally administered arbidol is rapidly absorbed and distributed quickly into tissues and organs. Therefore special consideration had to be given to a very low quantification limit of the parent compound and potential interference from the main metabolites when analyzing arbidol in plasma samples. The HPLC methods used so far did not include an internal standard and were not sensitive enough for

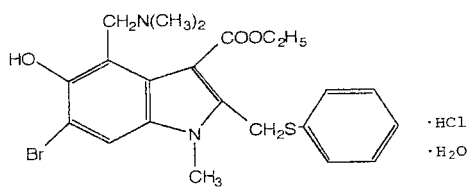

Fig. 1. Chemical structure of arbidol.

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the measurement of samples from a pharmacokinetic study in humans [1] or they were developed and validated for quality control aspects [2] and not for use with biological matrix. Therefore our objective was to develop a sensitive and specific HPLC method for determination of arbidol in human plasma.

The method was extensively validated fulfilling international guidelines [3].

2. Experimental

2.1. Chemicals and reagents

All chemicals and reagents used were of HPLC grade or analytical-reagent grade. Arbidol as well as the internal standard SI-5 {ethyl-6-bromo-5-hydroxy-1-methyl-2-[(phenylthio)methyl]-indole-3-carboxylate} were synthesized by G.D. Searle (Chicago, IL, USA). Sodium dihydrogenphosphate monohydrate, perchloric acid 70% as well as sodium hydrogencarbonate monohydrate (analytical-reagent grade) were purchased from Merck (Darmstadt, Germany). Solvents were of analytical and HPLC grade. *tert.*-Butyl methyl ether was of LiChrosolv quality and purchased from Merck as well as 0.1 *M* hydrochloric acid. Acetonitrile and methanol were of ChromAR quality (Promochem, Wesel, Germany). 1-Heptanesulfonic acid and ammonium perchlorate were purchased from Fluka (Buchs, Switzerland). Water purified by a Milli-Q system (Millipore, Bedford, MA, USA) was used in all procedures involving water.

2.2. Apparatus and chromatographic conditions

The HPLC apparatus consisted of a Kontron 420 pump (Kontron Instruments, Neufahrn, Germany), a GAT LCD 502 variable-wavelength UV detector (set at 315 nm) and a 710 B WISP autosampler (Waters, Eschborn, Germany). The integration was performed using the software Turbochrom 3 (Perkin–Elmer Nelson, Cupertino, CA, USA) installed on a IBM-compatible personal computer. The analytical column (stainless steel, 250×4.6 mm I.D.) was packed with 5 μm particles of LiChrosorb RP 8 (M. Grom, Herrenberg, Germany). Refilled columns were used throughout the validation procedures. As a guard column we used cartridges manufactured by Merck filled with LiChrospher RP 8, 5 μm (4×4 mm). Guard columns were replaced on a routine system after 60–90 injections (e.g., after one sequence) during the validation procedures. The columns were maintained at 35°C in a water bath. For analysis of plasma samples the composition of the mobile phase was 70% (v/v) methanol and 30% (v/v) of 5 m*M* 1-heptanesulfonic acid and 50 m*M* ammonium perchlorate with 1.32% (v/v, related to aqueous phase) triethylamine. The pH was adjusted to 3.0 with orthophosphoric acid. The flow-rate of the mobile phase was set to 1.2 ml/min.

2.3. Preparation of stock solutions, calibration levels and validation samples

Stock solutions of arbidol free base (2000 μg/ml) and the internal standard SI-5 (100 μg/ml) were prepared in acetonitrile. Calibration levels in the appropriate drug-free matrix were prepared yielding a concentration range from 0.005 μg/ml up to 10.2 μg/ml. Validation samples (VSs) at three different concentration levels were prepared leading to concentrations of 8.65, 0.866 and 0.055 μg/ml. Like the stock solutions all calibration levels and validation samples were divided into aliquots anticipating the number needed for the validation experiments and stored frozen at −20°C until analysis. The calibration samples and validation samples were worked up exactly the same way as unknowns.

2.4. Sample pretreatment

During sample pretreatment samples were kept protected from light. Plasma samples were thawed, thoroughly vortexed for 15 s and centrifuged for 1 min at 14 000 rpm. One thousand microliters of the centrifuged plasma sample were transferred into a brown glass tube and 250 μl of a saturated solution of sodium hydrogencarbonate were added to the plasma in order to adjust the pH value to pH 9. Next 100 μl of a solution containing the internal standard (5 μg/ml SI-5) were added. No internal standard solution was added to blank samples. Then 5 ml of *tert.*-butyl methyl ether were added to the samples. The mixture was shaken for 10 min at 50 rpm followed by 10 min of centrifugation at 4000 rpm to get a sharp separation between organic phase and

plasma. Thereafter the organic phase was completely transferred to another brown glass tube and evaporated to dryness at 35°C under a gentle flow of nitrogen. The residue was reconstituted in 200 μl mobile phase, transferred to an Eppendorf cap and centrifuged for 5 min at 14 000 rpm. The total solution was transferred to an Eppendorf 1.5 ml tube and 200 μl *n*-hexane were added. After mixing for 15 s the samples were centrifuged for 3 min at 14 000 rpm. The clear aqueous phase was transferred to an autosampler vial and a 100 μl aliquot was injected onto the HPLC system.

2.5. Validation of the assay

Accuracy and precision of the assay as well as the linearity of the calibration curve were determined intra-day and inter-day on five different days. Recovery of the analyte and the internal standard following the sample clean-up procedures relative to aqueous solutions were determined at different concentration levels. Stability of the analyte was tested at different light and temperature conditions in the appropriate biological matrix prior and subsequent to the sample preparation procedures. All experiments were performed in a total of six sequences.

3. Results and discussion

3.1. Specificity, linearity and sensitivity

The specificity of the method was determined by screening blank plasma of four different healthy donors. Arbidol sulfoxide, arbidol sulfone as well as SI-5 sulfoxide as potential metabolites, were investigated for their chromatographic behaviour to determine whether they interfere with arbidol or the internal standard. No other compounds were investigated as the assay was developed for use in studies with healthy volunteers. Since no suitable fluorescence of arbidol could be found, analysis of the UV spectra were performed. Maximum absorption values of UV light for arbidol as well as for SI-5 were found at 270 and 315 nm wavelength. We decided to validate the assay with a UV detection at 315 nm as we expected the molar absorption to be high enough for a sensitive determination combined with the advantage of a reduced risk of detecting coeluting endogenous compounds at the higher wavelength. Representative chromatograms of a spiked plasma sample (0.08 μg/ml arbidol free base) and plasma samples derived from a healthy volunteer prior to administration of arbidol are shown in Fig. 2 and Fig. 3. Retention times for arbidol were between 5.3–5.4 min and between 8.5–8.8 min for the internal standard SI-5. Arbidol and the internal standard are completely resolved from each other as well as from any endogenous peaks in plasma. Arbidol sulfoxide showed a retention time of 3.5 min and hence no potential interference with the two compounds of interest. Arbidol sulfone, the other putative metabolite, eluted even before arbidol sulfoxide as proved by gradient elution.

For evaluation of the calibration graph a weighted linear regression ($1/x$) was performed with nominal concentrations of calibration levels and measured peak height ratios (peak height analyte/peak height internal standard). The slope and intercept of the 12-point regression graph were determined according to standard equations. A total of ten calibration

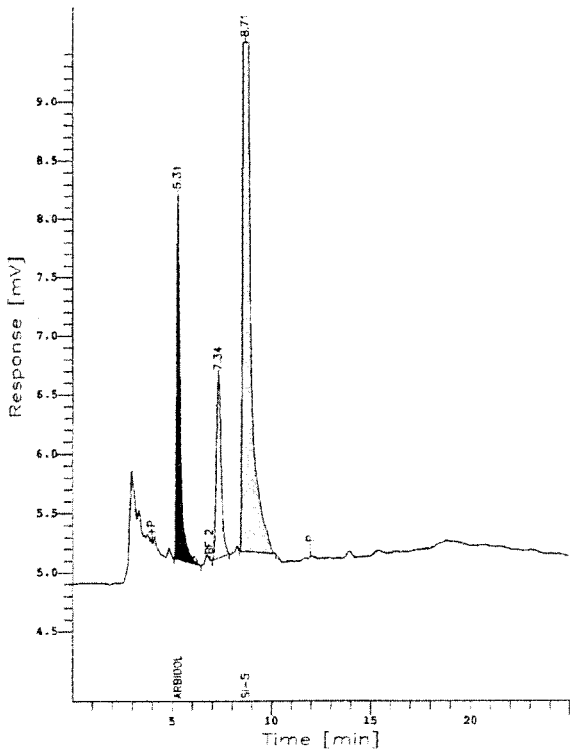

Fig. 2. Blank plasma spiked with 0.0794 μg/ml arbidol free base.

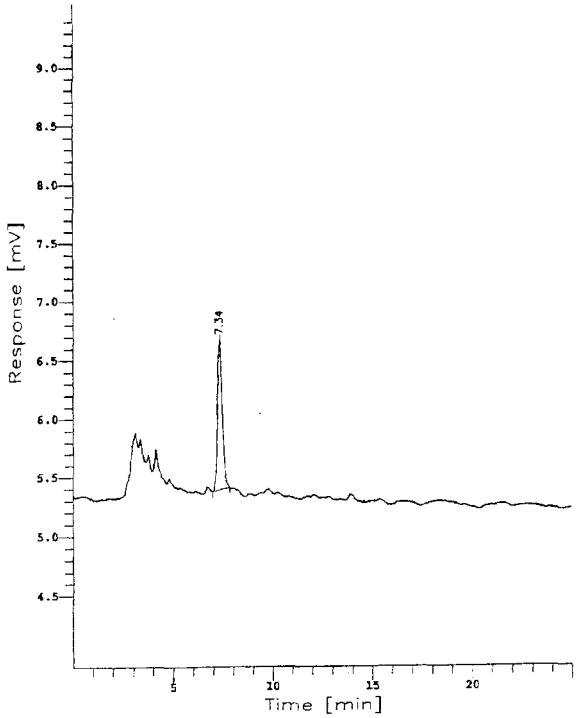

Fig. 3. Blank plasma of a healthy volunteer prior to drug administration.

curves were assayed and calculated on five different days. Linearity of the assay could be shown over a concentration range of 0.005–10.2 μg/ml. The coefficient of correlation (r^2) was above 0.999 in each case. The intercept relative to the response of the lowest calibration level ranged between 1.9–15.4% for the individual curves with a mean value of 9.4% (Table 1). The relative errors of the individual calibration points were between −4.9 and 14.9%. The mean accuracy of the different calibration levels were between 1.0% (calibration level No. 4: 1.27 μg/ml arbidol free base) and 7.9% (calibration level No. 12: 0.005 μg/ml arbidol free base).

The lowest calibration level used in the validation calibration curve yielded a concentration of 0.005 μg/ml arbidol in plasma. The individual values of the relative error of this calibration level were between −2.8 and 14.9%. Therefore this calibration level was chosen as the limit of quantification.

3.2. Precision and accuracy

The intra-day precision and accuracy were determined by analyzing five aliquots of each validation sample within one sequence. Means, standard deviations, relative standard deviation (R.S.D.) values and relative error (R.E.) values were determined. The intra-day precision was high and in a narrow range with values of 2.7% ($n=10$) for a concentration of 8.65 μg/ml arbidol, 2.5% ($n=10$), and 4.0% ($n=10$) for a concentration of 0.87 μg/ml, and 0.05 μg/ml arbidol, respectively (Table 2). Inter-day variation was determined on five different days at the same concentration levels as intra-day. The mean value of the inter-day precision of the highest validation sample was 3.3% ($n=20$), the values for the medium and lower validation samples were 1.6%

Table 1
Calibration parameters of all sequences

Parameter	Analyzed on Day									
	1 Cal. curve 1	1 Cal. curve 2	2 Cal. curve 3	2 Cal. curve 4	3 Cal. curve 5	3 Cal. curve 6	4 Cal. curve 7	4 Cal. curve 8	5 Cal. curve 9	5 Cal. curve 10
Number of levels used for linear regression	12	12	12	11	12	10	11	11	12	10
Slope	3.07	3.08	2.97	2.93	2.98	3.05	3.06	3.10	2.95	2.91
Intercept	0.00225	−0.00156	0.00225	0.00136	0.00225	−0.00142	−0.00720	0.00145	-0.000668	0.00818
Coefficient of correlation r^2	0.9997	0.9989	0.9991	0.9992	0.9998	0.9987	0.9997	0.9989	0.9997	0.9995
Response of lowest level	0.0161	0.0140	0.0139	0.0146	0.0149	0.0140	0.0149	0.0173	0.0139	0.0593
Intercept: % of response of lowest level	14.0	11.1	9.78	15.4	1.91	10.1	4.38	8.38	4.81	13.8

Table 2
Intra-day and inter-day precision of validation samples

	VS 1 8.65 μg/ml[a] Conc. (μg/ml)	VS 2 0.866 μg/ml[a] Conc. (μg/ml)	VS 3 0.0547 μg/ml[a] Conc. (μg/ml)
Intra-day			
Mean	8.42	0.875	0.0553
S.D.	0.23	0.022	0.0022
R.S.D. (%)	2.73	2.51	3.98
Inter-day			
Mean	8.60	0.888	0.0566
S.D.	0.28	0.014	0.0015
R.S.D. (%)	3.26	1.58	2.65

[a] Nominal arbidol concentration (μg/ml).

($n=20$) and 2.7% ($n=20$) (Table 2). These values were in a similar range or even lower as when determined within one run. The accuracy was calculated as the R.E. by compiling the measured concentrations with the nominal concentrations of arbidol in validation samples. For the intra-day accuracy the individual deviations ranged from −7.5 to 1.3% for the high validation sample (8.65 μg/ml arbidol free base), −1.7 to 5.7% for the medium validation sample (0.87 μg/ml arbidol free base), and −3.3 to 9.0% for the low validation sample (0.05 μg/ml arbidol free base). The respective values of the inter-day accuracy confirmed the range of the intra-day values. The individual values ranged from −7.5 to 6.7%, −0.2 to 5.7%, and −3.3 to 8.0% for the high, medium, and low validation samples, respectively.

3.3. Recovery

Recovery was measured by comparison of peak heights of non-extracted standards in mobile phase versus extracted standards of spiked plasma. The mean absolute recovery of arbidol in plasma was found to be 96.3 and 100.6% at 1.00 and 10.0 μg/ml, respectively. The mean recovery of the internal standard at 0.50 μg/ml was found to be 95.2%.

3.4. Stability experiments

Evaluation of stability samples was in general based on the comparison of the respective sample at the respective condition with an initial value.

Light stability of arbidol in plasma (10.6 μg/ml) was determined under the following conditions: (A) not light protected, stored in direct sunlight at a laboratory window; (B) not light protected, stored at ambient room light in the laboratory; (C) light protected.

Arbidol free base in plasma was found to be light sensitive when exposed to intensive daylight, e.g., sunlight. We observed a degradation of arbidol leading to a loss of 46% in a 6 h time period when exposed to sunlight. There is no sensitivity to ambient light exposure when compared to control plasma (Table 3). All other conditions tested showed no influence on the stability of arbidol. For the determination of the counter stability (stability during sample preparation) validation samples were thawed at certain timepoints according to a time schedule (24, 6, 5, 4, 3, 2 and 1 h before sample clean-up). After thawing one aliquot was stored at room temperature and the other one was stored at +4°C. During storage the samples were protected from light. At zero time all samples were prepared and measured. Stability of arbidol in native plasma at +4°C and at room temperature was given over a 24 h period (Table 4). Stability was also tested after sample preparation at −20°C and ambient temperature after 24 and 72 h storage. No instability of arbidol was observed after sample clean-up over a

Table 3
Light stability of arbidol free base at ambient temperature

Treatment	Storage time (h)	Mean conc.[a] (μg/ml)	Stability (%)
Initial	0	10.6	100.0
Sunlight	2	8.19	77.3
	4	7.15	67.5
	6	5.72	54.0
Ambient room light	2	10.4	98.1
	4	10.5	99.1
	6	10.2	96.2
	24	9.37	88.4
Light-protected	2	10.3	97.2
	4	10.1	95.3
	6	10.2	96.2
	24	9.63	90.8

[a] Nominal arbidol concentration (μg/ml).

Table 4
Counter stability of arbidol (VS2) at room temperature and at 4°C (light-protected)

Storage (h)	Room temperature		4°C	
	Conc. (μg/ml)	Stability (%)	Conc. (μg/ml)	Stability (%)
0	0.866[a]	100.0	0.866[a]	100.0
1	0.893	103.1	0.879	101.5
2	0.899	103.8	0.880	101.6
3	0.908	104.8	0.861	99.4
4	0.887	102.4	0.893	103.1
5	0.904	104.4	0.899	103.8
6	0.877	101.3	0.910	105.1
20	0.831	96.0	0.892	103.0
24	0.862	99.5	0.885	102.2

[a] Nominal arbidol concentration (μg/ml).

time period of 72 h (Table 5). Also four freeze–thaw cycles did not significantly alter the amount of arbidol analyzed in the spiked plasma samples (Table 6). Thus, it was concluded that arbidol is stable for a sufficient time period after thawing prior to sample preparation and after sample preparation. Strict light protection is required during the whole process of analysis.

4. Application

The validated method was used to analyze 1000 plasma samples from a pharmacokinetic study in healthy volunteers. Calibration levels were identical to the calibration levels used during the validation procedures. Samples were analyzed during a three month period in 28 sequences. The values obtained for the calibration levels and the resulting calibration graphs were in a similar range as observed during validation. The coefficient of correlation (r^2) was above 0.999 in each case. The intercept expressed relative to the response of the lowest calibration level ranged between −22 and 26%. The relative errors of the individual calibration points was between −11 and 12% (at a concentration of 0.005 μg/ml). The inter-day precision of the quality controls were very similar to those observed during validation. The precision of the high quality control was 4.1% (n=34) and the low quality control yielded a value of 5.9% (n=35). A representative plasma curve of a volunteer obtained after a single oral dose of 200 mg arbidol is shown in Fig. 4.

Table 6
Freeze–thaw stability of arbidol in plasma

Cycle	Conc. (μg/ml)	Mean (μg/ml)	Stability (%)
0	10.2		
Initial	10.7 10.9	10.6	100.00
1	11.4 10.8	11.1	104.7
2	11.4 10.4	10.9	102.8
3	10.3 10.4	10.4	98.1
4	10.6 10.5	10.6	100.0

5. Conclusions

This paper describes a sensitive, selective and reliable HPLC assay for arbidol in human plasma. The limit of quantification was significantly improved compared to a previous method. Putative metabolites do not interfere with the analyte or

Table 5
Stability of arbidol after sample clean-up

Treatment	Time (h)	Conc. (μg/ml)	Stability (%)	Conc. (μg/ml)	Stability (%)
Control	0	8.65[a]	100.0	0.866[a]	100.0
Storage at +4°C	24	8.87	102.5	0.905	104.5
	48	8.75	101.2	0.891	102.9

[a] Nominal arbidol concentration (μg/ml).

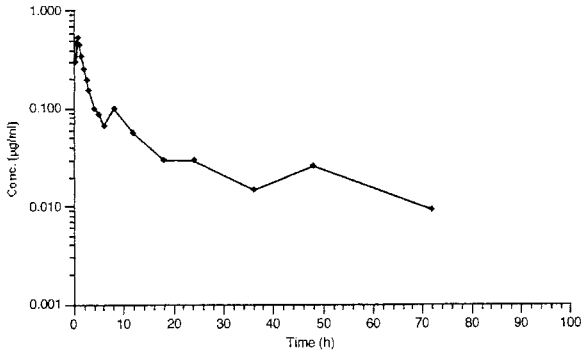

Fig. 4. Plasma levels of arbidol free base in a healthy volunteer after a single oral dose of 200 mg arbidol.

internal standard. The method includes a liquid–liquid extraction of drug and internal standard from plasma with *tert.*-butyl methyl ether followed by evaporation and reconstitution in mobile phase. Before injection, lipophilic interferences are removed from the reconstituted sample by extraction with *n*-hexane. Excellent separation of the analyte was achieved in a reversed-phase system by addition of ion-pair reagent to the mobile phase. Since arbidol exhibited no suitable fluorescence UV absorbance at a wavelength of 315 nm was used for detection.

A linear quantification range from 0.005–10.2 µg/ml for plasma (three orders of magnitude) could be established. The method has been successfully used for measurement of samples derived from a human pharmacokinetic study after single oral dosing of arbidol at different doses (50–1000 mg). Plasma levels could be followed up until 48 h after dosing depending on the dose given. The precision and accuracy as found during the validation procedure for plasma was in total confirmed during the routine analysis. The method proved to be suitable and reliable for the determination of basic pharmacokinetics of this novel antiviral compound even at low doses like 50 mg.

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