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Journal of Chromatography B, 686 (1996) 229–234

JOURNAL OF CHROMATOGRAPHY B: BIOMEDICAL APPLICATIONS

Simultaneous determination of GTS-21 and its metabolite in rat plasma by high-performance liquid chromatography using solid-phase extraction

Ryotaro Azuma*, Yoshinori Minami, Toshiyuki Satoh

Pharmacokinetics Research Laboratory, Taiho Pharmaceutical Co., Ltd., 224-2, Ebisuno, Hiraishi, Kawauchi-cho, Tokushima 771-01, Japan

Received 12 March 1996; revised 20 May 1996; accepted 21 May 1996

Abstract

It has been suggested that GTS-21 can improve the learning deficits and inhibit the neuro-degeneration in patients with Alzheimer's disease. This paper describes a reversed-phase high-performance liquid chromatographic assay with visible detection at 405 nm for determination of GTS-21 and its metabolite, 4-hydroxy-GTS-21 in rat plasma. The method uses solid-phase extraction with a Bond Elut C_{18} column. A quantitation limit of 1.0 ng/ml was achieved using 0.5 ml of rat plasma. In the validation study, the coefficients of variation and the relative errors of each compound were less than 10%. Also freeze–thaw and storage stability were confirmed. This method has proved to be applicable to the pharmacokinetic study of GTS-21 in rats.

Keywords: 3-(2,4-Dimethyloxybenzylidene)anabaseine; Nicotinic acetylcholine receptor agonist

1. Introduction

GTS-21, 3-(2,4-dimethyloxybenzylidene)-anabaseine dihydrochloride (I, Fig. 1), an agonist for nicotinic acetylcholine receptors, has been suggested to improve learning deficits and to inhibit the neuro-degeneration in animal models, and its efficacy for Alzheimer's disease has been suggested [1–3].

Compound I which has a novel anabaseine skeleton is currently developing as an oral drug, and its effective plasma concentration in rat is expected to be of the ng/ml order. Therefore, a high-sensitivity analytical method for determination of I is re-

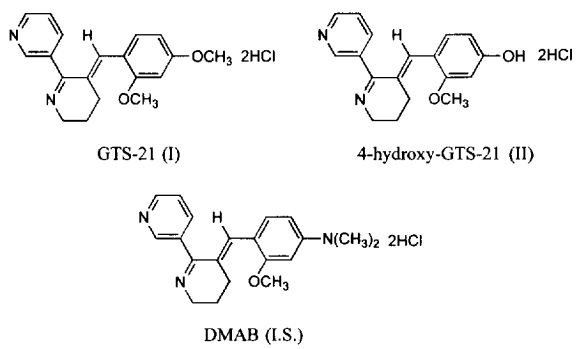

Fig. 1. Structures of GTS-21 (I), 4-hydroxy-GTS-21 (II) and DMAB (I.S.).

*Corresponding author.

0378-4347/96/$15.00
PII S0378-4347(96)00233-2

quired. The anabaseine derivative usually does not have a visible absorption; however, I and its metabolites have a visible absorption. Since visible detection, which has a high selectivity, can be used, a rapid and simple sample preparation without complications would be anticipated. Due to its easy handling, solid-phase extraction is a convenient and powerful tool for sample clean-up. It is widely used in the analysis of drugs in biological samples.

Compound I is metabolically changed to 4-hydroxy-GTS-21 (II, Fig. 1) by oxidative O-demethylation at the 4-position of the aromatic ring in rats. Moreover, this metabolite is converted to form glucuronide by conjugation. 4-Hydroxy-GTS-21, which retains its agonist activity, may contribute to the pharmacological effect after administration of GTS-21. Thus, to characterize the pharmacokinetic parameters of both compounds, we have developed a method for their simultaneous determination.

This report describes the methodology and validation of a high-performance liquid chromatographic assay for the simultaneous determination of I and II in rat plasma. This method is highly sensitive and highly selective because of the visible detection and it is simple owing to the use of solid-phase extraction.

2. Experimental

2.1. Chemicals and reagents

Compound I and DMAB (Fig. 1), used as the internal standard (I.S.) were supplied by Kobe Natural Products and Chemicals (Kobe, Japan). Compound II was supplied by Taiho Fine Chemicals (Saitama, Japan).

HPLC-grade acetonitrile and methanol were purchased from Kanto Chemical (Tokyo, Japan). The water used was purified with the Milli-Q system (Millipore, Tokyo, Japan). Guaranteed reagent acetic acid, and potassium dihydrogen phosphate (KH_2PO_4) were purchased from Nakarai Tesque (Kyoto, Japan). Special-grade phosphoric acid and first-grade ammonium hydroxide were purchased from Wako (Osaka, Japan).

2.2. Chromatography

The HPLC system (Shimadzu, Kyoto, Japan) comprised an ERC-3512 on-line degasser (Erma, Tokyo, Japan), two LC-10AD pumps, an SIL-10A auto-injector, a CTO-10A column oven, an SPD-10AV UV-VIS detector and a workstation CLASS-LC10.

Separation was performed at 40°C on an Inertsil ODS-3 (150×4.6 mm I.D., 5 μm, GL Sciences, Tokyo, Japan) with a Baked Metal Filter (2 μm, Shimadzu, Kyoto, Japan) as a guard column. The visible wavelength was set at 405 nm. Gradient elution was performed at a flow-rate of 1.0 ml/min using a mobile phase of 10 m*M* KH_2PO_4 (adjusted with phosphoric acid to pH 2.8)–acetonitrile. The linear gradient ran from 15 to 33% acetonitrile in 18 min. The total run time was 30 min with a post-run time of 11 min for re-equilibration. The HPLC rinse solution consisted of acetic acid–water–acetonitrile (2:48:50, v/v). Prior to use, it was degassed using an aspirator.

As all analytes are highly photosensitive, the auto-injector was covered with aluminum foil.

2.3. Extraction

The extraction procedure was carried out under yellow fluorescent light.

Bond Elut C_{18} extraction columns (200 mg/3.0 ml, Varian, Harbor City, CA, USA) were mounted on a Vac Elut SPS 24 (Varian). Prior to use, the columns were activated with 2.5 ml of methanol, water and 50 m*M* KH_2PO_4 (pH 3.0). Aliquots of 0.5 ml centrifuged (1200 *g*, 10 min) plasma were mixed with 0.1 ml of 2 μg/ml I.S. and 0.5 ml of 50 m*M* KH_2PO_4 (pH 3.0). The mixture was loaded on C_{18} columns and drawn through under vacuum. The columns were rinsed with 2.5 ml of water, followed by 2.5 ml of water–methanol (5:5, v/v). The compounds were eluted twice with 2.0 ml of 0.25% ammonium hydroxide in methanol (v/v). The eluent was dried under nitrogen at 40°C, the residue was resolved with 0.2 ml of 10 m*M* KH_2PO_4 (pH 2.8)–acetonitrile (85:15, v/v). The solution was centrifuged at 1200 *g* for 5 min and 75 μl of the supernatant was injected into the HPLC system.

2.4. Standards and calibration curve

All standard solutions were put in amber volumetric flasks. Stock solutions of each of the compounds were initially prepared at a concentration of 100 μg/ml in 10 m*M* KH_2PO_4 (pH 3.0). From these solutions, calibration-standard solutions containing two compounds were prepared by sequential dilution to give 5000, 1000, 250, 50, 10 and 5 ng/ml solution of each compound. The I.S. solution was prepared by the same solvent and diluted to 2 μg/ml. A calibration curve was prepared by spiking plasma with 0.1 ml of standard solutions ranging from 1 to 1000 ng/ml of plasma.

Quantification was accomplished based on the peak-height ratio of drug to I.S. using CLASS-LC10. The $1/conc^2$ weighted least-squares regression line was calculated.

2.5. Recovery, precision and accuracy

The absolute extraction recovery was calculated at four concentrations for each compound, at one concentration for I.S. The peak heights of extracted samples were compared with those of unextracted standard solutions.

Intra- and inter-assay (each, $n=5$) were evaluated by spiking plasma with four different concentrations. The precision was expressed as a coefficient of variation (C.V.) by calculating the standard deviation as a percentage of the mean found concentration. The accuracy was expressed as a relative error (R.E.) by calculating the found concentration as a percentage of the added concentration. Linear regression analysis was performed to calculate the slope, the intercept and the correlation coefficient (r) of the calibration curve.

2.6. Freeze–thaw and storage stability

Blank rat plasma was spiked with a standard solution of each compound to give final concentrations of 5 and 50 ng/ml (each, $n=3$). For freeze–thaw stability tests, these aliquots of plasma were frozen at −80°C. Next day these samples were thawed and then frozen again. After 2 days these samples were thawed and prepared for analysis. For storage stability tests, plasma samples were stored at −80°C. After 64 and 118 days these samples were thawed and prepared for analysis. The stability was evaluated as à R.E. compared with the theoretical value.

2.7. Application

Male Sprague–Dawley rats ($n=4$ or 5), 250–300 g, were fasted overnight 12 h prior to and 8 h after dosing. Rats were given I (3 mg/kg) orally through a gastric tube. For oral administration, I was dissolved in Milli-Q water, and given in a volume of 5 ml/kg. At 0.5, 1, 2, 3, 4, 6, 8, 12, 24 and 48 h after administration, rats were killed by taking the blood from the descending aorta with a heparinized syringe under anaesthesia with ether. The blood was transferred into amber acrylic tubes. Plasma was immediately separated by centrifugation at 1200 *g* for 15 min and stored frozen at −80°C before analysis.

Non-compartment methods were applied for the pharmacokinetic analysis. The concentration in plasma (C_{max}) was obtained by inspection of the mean concentration–time curves. The area under the curve (AUC_{0-inf}) was calculated according to the linear trapezoidal rule. The elimination half-life ($t_{1/2}$) was calculated with the last three points by linear regression analysis.

3. Results and discussion

3.1. Chromatography

To minimize the tailing factor and to obtain a good resolution of I and I.S., the pH of the mobile phase was carefully chosen. At pH 2.8 the tailing factors for all compounds were less than 1.3. Under these conditions the resolution of I and I.S. was about 4.0, providing sufficient separation. Since with more acidic mobile phases the resolution decreased, a mobile phase pH of 2.8 was selected. Among several ODS columns, the analytical column selected was an Inertsil ODS-3, giving the smallest tailing factor. To prevent contamination after injection, the HPLC rinse solution included acetic acid. Fig. 2A shows a typical chromatogram of a blank plasma

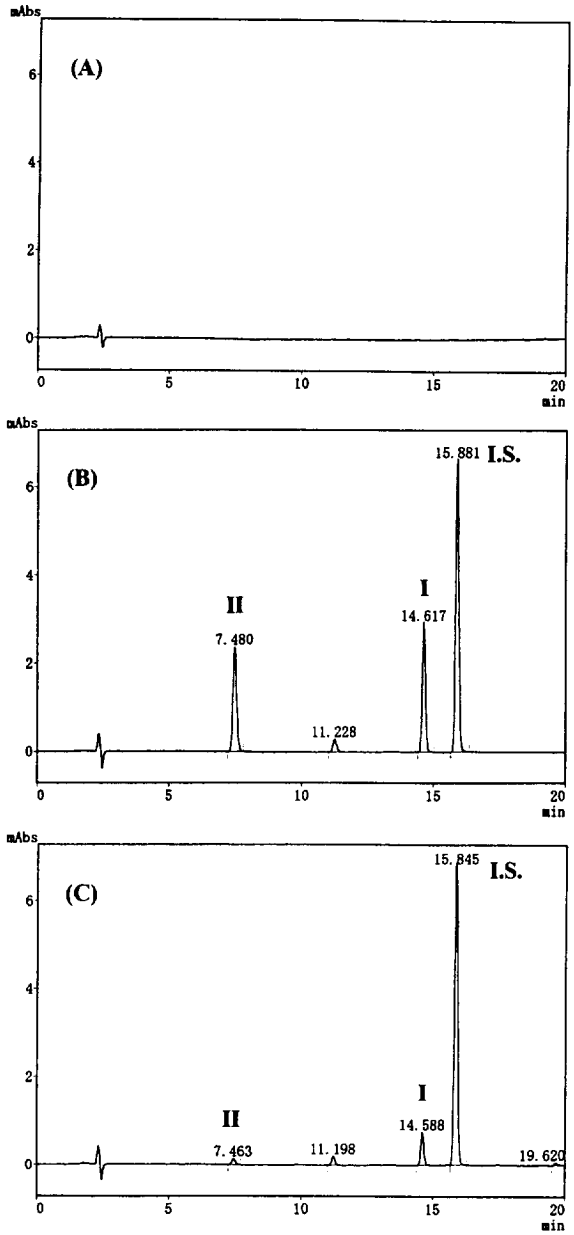

Fig. 2. Typical chromatograms of a blank plasma sample (A), a plasma spiked with 50 ng/ml of I, 50 ng/ml of II and 400 ng/ml of I.S. (B), a plasma sample obtained 2 h after oral administration (C).

sample. No interference peaks are found at the elution times of I, II and I.S., and no endogenous biological material was observed in this chromatogram. Fig. 2B shows a blank plasma spiked with 50 ng/ml of I, 50 ng/ml of II and 400 ng/ml of I.S. Compound II, I and I.S. were eluted at 7.5, 14.6 and 15.9 min, respectively. The peak at 11.2 min was an impurity originating from the I.S. Although the retention times vary approximately 1 min from one lot to another, resolution is not affected at all. Moreover, as long as the same column is used, the retention times do not change during an analytical run.

The analytical column pressure gradually increased after 200–300 injections of plasma extracts. This pressure increase was attributed to accumulation of particles from the plasma at the column frit. Normal pressure was restored by either changing the baked metal filter or the analytical column joint at the beginning of the run. This operation enables the determination of more than 1000 samples using one column.

3.2. Extraction

An efficient SPE method is beneficial to assay precision and accuracy. Although the C_{18} column is the most popular among the various extraction columns, it is not very selective because many isolated molecules can be retained [4]. However, with the selection of an appropriate eluent and with the highly selective detection this drawback can be overcome. We first tried methanol as an eluent; however, the recovery of I was not satisfactory. The recovery could be improved by adding 0.25% of ammonium hydroxide. The mean extraction recovery and the S.D. from the plasma samples for each compound are shown in Table 1. The overall re-

Table 1
Extraction absolute recovery of I, II and I.S.

Compound	Concentration added (ng/ml)	Recovery (mean±S.D.) (%)
I (n=5)	1	99.4±5.2
	2	86.5±4.9
	50	86.0±8.9
	1000	88.3±8.8
II (n=5)	1	86.8±8.7
	2	77.8±3.6
	50	83.9±8.6
	1000	85.7±7.7
I.S. (n=20)	400	82.3±6.5

covery rates were 77–100% in plasma. The recovery of II tended to be slightly lower than that of I.

3.3. Validation

Calibration curves were linear over the range 1–1000 ng/ml for rat plasma. From seven assay runs, linear regression equations for I and II were, respectively, $y=(120.15\pm3.16)x-(0.05751\pm0.05051)$ and $y=(156.33\pm5.50)x+(0.06363\pm0.03411)$, where y is the drug concentration and x is the peak-height ratio of drug to I.S. The r for I and II were, respectively, computed as 0.99936 ± 0.00037, 0.99927 ± 0.00055.

The intra- and inter-assay precision and accuracy for I and II are shown in Table 2. They were evaluated by analysing five replicates of four different samples (1, 2, 50 and 1000 ng/ml) containing each compound. The C.V. and the R.E. at 50 and 1000 ng/ml were less than 5%, indicating a very small error. At 1 and 2 ng/ml these values were less than 10%.

Freeze–thaw and storage stability tests were examined at concentrations of 5 and 50 ng/ml. After two freeze–thaw cycles, the found mean values and the S.D. for each compound are shown in Table 3. Also, the results of the storage stability test are shown in Table 3. The data suggested that the freeze–thaw process and storage for 118 days have no effect on the determination of I and II.

3.4. Application to pharmacokinetic study

The present method has been applied in the pharmacokinetic study of I in rats after oral administration of 3 mg/kg. A typical chromatogram from rat plasma sample is shown in Fig. 2C. Fig. 3 shows mean plasma concentration–time profiles of I and II. The analytical method was sensitive to parent drug and could measure concentration levels up to 8 h post dose. The levels of II were very low; some of the four or five samples were below the quantitation limit except for those at 2 and 3 h. Even though the concentration was low, the existence of II was confirmed in all samples up to 4 h post dose. The pharmacokinetic parameters were calculated from the mean data; C_{max}, AUC_{0-inf} and $t_{1/2}$ for I were, respectively, 19.93 ng/ml, 79.23 ng h/ml and 1.34 h. In contrast, C_{max} for II was 2.39 ng/ml.

For analysis of unknown samples, we always determined quality control (QC) samples of three

Table 2
Precision and accuracy of I and II analyzed from rat plasma ($n=5$)

Compound	Concentration added (ng/ml)	Concentration found (mean±S.D.) (ng/ml)	C.V. (%)	R.E. (%)
I				
Intra-assay	1	1.06±0.04	3.8	+6.3
	2	1.96±0.07	3.6	−2.2
	50	50.08±0.40	0.8	+0.2
	1000	1007.82±16.69	1.7	+0.8
Inter-assay	1	0.98±0.08	8.2	−1.8
	2	1.98±0.12	6.1	−1.1
	50	50.48±0.55	1.1	+1.0
	1000	1027.41±26.83	2.6	+2.7
II				
Intra-assay	1	0.97±0.08	8.2	−2.8
	2	1.81±0.07	3.9	−9.6
	50	50.21±0.34	0.7	+0.4
	1000	1001.62±32.50	3.3	+0.2
Inter-assay	1	1.10±0.07	6.4	+9.9
	2	2.18±0.12	5.5	+8.8
	50	51.15±2.02	3.9	+2.3
	1000	1024.43±50.88	5.0	+2.4

Table 3
Freeze–thaw and storage stability of I and II in rat plasma ($n=3$)

Compound	Concentration added (ng/ml)	Concentration found (mean±S.D.) (ng/ml)	C.V. (%)	R.E. (%)
I				
Freeze–thaw	5	5.02±0.16	3.2	+0.4
	50	51.19±0.30	0.6	+2.4
Storage day 64	5	5.05±0.20	4.0	+1.0
	50	50.56±0.90	1.8	+1.1
Storage day 118	5	4.99±0.06	1.2	−0.2
	50	48.08±0.43	0.9	−3.8
II				
Freeze–thaw	5	5.25±0.03	0.6	+5.0
	50	52.14±1.13	2.2	+4.3
Storage day 64	5	5.45±0.24	4.4	+9.0
	50	50.52±2.54	5.0	+1.0
Storage day 118	5	5.18±0.22	4.2	+3.6
	50	50.20±0.66	1.3	+0.4

concentrations (5, 50 and 500 ng/ml) at the beginning and at the end of the run. All relative errors of found values were less than 10% (data not shown). Thus, we concluded that there is no problem of stability in the auto-injector vials.

4. Conclusion

This report describes the determination of GTS-21 and 4-hydroxy-GTS-21 using solid-phase extraction and reversed-phase HPLC with visible detection. This method has a high selectivity and high sensitivity with a quantitation limit of 1.0 ng/ml using 0.5 ml of rat plasma. All coefficients of variation and relative errors obtained for this method were less than 10%. GTS-21 and 4-hydroxy-GTS-21 are stable in rat plasma at −80°C for at least 118 days.

Originally this method had been developed for Phase I clinical studies in human plasma. The applicability to the analysis of rat plasma was demonstrated.

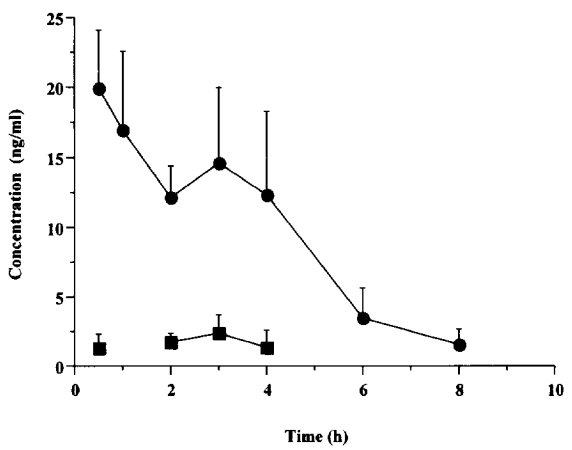

Fig. 3. Plasma concentration–time profiles of I (●) and II (■) in rats after oral administration of 3 mg/kg of GTS-21.

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