High-Performance Liquid Chromatographic Determination of Yohimbine in Serum by Chemiluminescence Detection

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An ion-pair high-performance liquid chromatography with chemiluminescence detection using tris(2,2'-bipyridyl)ruthenium(II) [Ru(bpy)₃²⁺] was developed for the determination of yohimbine (YOH) in human serum. A sample solution, containing narceine as an internal standard, was treated with a Bond-Elut Certify disposable column, and the purified fraction was separated on a TSKgel ODS-80Ts column. The recovery of YOH at 50 ng/ml was 97.2±4.4% (mean±SD, *n*=5). The detection limit was calculated to be 30 ng/ml of YOH in serum (*S/N*=3). The method was successfully applied to drug monitoring of the YOH level in the serum of healthy volunteers.

Keywords Yohimbine, serum, high-performance liquid chromatography, chemiluminescence, ruthenium

Yohimbine (YOH), methyl 17 α -hydroxy-yohimban-16 α -carboxylate, is an indole alkaloid obtained from various botanical sources. It has been studied for over 100 years, and is popularly believed to have aphrodisiac properties. YOH is an α_2 -adrenergic antagonist¹ and has been used to study α_2 -adrenoreceptors in humans.² As might be expected for this interesting compound, several chromatographic methods have been reported for the determination of YOH.³⁻⁸ In these methods, either a fluorescence detector or an electrochemical detector is employed because of its high sensitivity and selectivity.

Recently a chemiluminescence (CL) detection system using tris(2,2'-bipyridyl)ruthenium(II), Ru(bpy)₃²⁺, as a chemiluminescent reagent has been shown to be a sensitive and selective detection technique in high-performance liquid chromatography (HPLC) and flow injection analysis for the determination of aliphatic and alicyclic tertiary amines⁹⁻¹³, oxalate¹⁴⁻¹⁶ and indoles.¹⁷ For the preparation of unstable Ru(bpy)₃³⁺ from stable Ru(bpy)₃²⁺, three methods were reported using a batch electrochemical reactor¹³, an on-line electrochemical reactor¹⁷ and an electrogenerated CL flow cell. More recently, Yamazaki *et al.* showed that an on-line photochemical reactor could be applicable for the purpose of preparation.¹⁸ As shown in Fig. 1, YOH has a tertiary amine and an indole moiety; therefore, HPLC with CL detection using the ruthenium complex can be expected to be applicable for determining YOH.

We described here a HPLC method for the determination of YOH in serum with CL detection after solid-phase extraction of the analyte. This method was applied to pharmacokinetic studies of YOH in healthy volunteers.

Experimental

Materials

YOH, narceine (NAR), tris(2,2'-bipyridyl)ruthenium (II) chloride hexahydrate [Ru(bpy)₃Cl₂·6H₂O], potassium peroxodisulfate (K₂S₂O₈) were purchased from Aldrich Chemical Co. (Milwaukee, WI, USA). HPLC-grade methanol and water were purchased from Tokyo Kasei Co. (Tokyo, Japan). All other reagents were of highest grade commercially available. The mobile phase of HPLC was passed through a 0.45- μ m filter prior to use. A Bond-Elut Certify disposable column was purchased from Varian Co. (Harbor City, CA, USA).

Apparatus and operating conditions

The HPLC-CL system consisted of two HPLC pumps (JASCO Model PU-880, JASCO, Co. Ltd., Tokyo, Japan), a photo-reactor assembled with an ultraviolet lamp (4 W), a coiled PTFE tube (0.5 mm i.d.×8 m), an

Fig. 1 Chemical structures of yohimbine and narceine.

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S-3400 chemiluminescence detector (flow cell, 100 μ l, Soma Kogaku, Hinode, Tokyo, Japan), a Rheodyne Model 7125 injector (Rheodyne, Inc., Cotati, CA, USA) with an effective volume of 20 μ l and an LR4220 recorder (Tosoh, Co. Ltd., Tokyo, Japan). A TSKgel ODS-80Ts (4.6 mm i.d. $\times$ 150 mm, Tosoh) was used as the analytical column with a guard-column of TSKguardgel ODS-80Ts (3.2 mm i.d. $\times$ 15 mm, Tosoh). The column was maintained at 40°C by a CO-8020 column oven (Tosoh). A mixture of methanol and 25 mM phosphate buffer solution (pH 3.5) (45:55, v/v) containing 10 mM sodium 1-heptanesulfonate was used as a mobile phase. The reagent solution, 25 mM phosphate buffer (pH 3.5) containing 1 mM Ru(bpy)₃Cl₂ and 1 mM K₂S₂O₈, was stored in a cooler (5°C). Although a 2 mM Ru(bpy)₃Cl₂ solution gave a somewhat greater CL signal, the 1 mM solution was used to save the reagent. The flow-rate used for these experiments was 0.5 ml/min.

Extraction procedure

To 1 ml of serum (or standard solution) placed in a 15 ml teflon-lined screw-capped culture tube, 1 ml of internal standard solution (NAR: 25 ng/ml) and 3 ml of 100 mM phosphate buffer (pH 6.0) were added. After mixing for 5 min, the contents were applied to a Bond-Elut Certify disposable column, which was conditioned by passing 2 ml of 100 mM phosphate buffer (pH 6.0) followed by 4 ml of methanol before use. The column was washed with 1 ml of water, 0.5 ml of 1 M acetic acid and 0.1 ml of methanol. The fraction of interest was then eluted with 2 ml of a 2% methanolic ammonia solution. The fraction was evaporated to dryness under a stream of nitrogen gas at 40°C. The residue was dissolved in 0.25 ml of the mobile phase; 20 μ l of this solution was injected onto the HPLC apparatus.

Standard solutions and calibration

Known amounts of YOH in the range 50 – 1000 ng/ml were added to blank serum samples and treated according to the described procedure. A calibration graph was constructed by plotting the peak height ratio of YOH to NAR (I.S.) against the concentration of YOH.

Recovery test for YOH

The spiked samples were prepared by adding 50, 500, and 1000 ng/ml of YOH to the blank serum. The solid-phase extraction and HPLC were carried out as described above. The recovery was determined by comparing the peak heights obtained from samples with those obtained after direct injection of YOH dissolved in the mobile phase.

Application to pharmacokinetics studies

The method was used to quantitate the serum concentration of YOH in a single-dose pharmacokinetic study. The subjects were two healthy male volunteers who gave a written, informed consent. Two subjects took 12

mg of Sutorupin M brand of YOH (Matsuda) in a fasting state. A 5-ml sample of blood was drawn at 0.25, 0.5, 1, 1.5, 2, 3 and 4 h after oral administration. Serum was separated by centrifugation at 3000g for 15 min and stored at –20°C until analysis. The total area under the observed serum concentration–time curve (AUC) was calculated by using the linear trapezoidal rule. A terminal elimination half-life was obtained directly from the one-compartment model. The peak serum level ($C_{\max}$) was obtained directly from the profile.

Results and Discussion

The optimum pH for the CL reaction of YOH was determined by FIA. The change in the CL intensity vs. pH is shown in Fig. 2. YOH was found to be most reactive at pH 3.5, which was also the optimum range for indoles.¹⁷ The NAR response was increased at much higher pHs. Burune and Bobbitt reported that the observed CL for amino-acids is dependent upon the pHs¹³, and that the CL intensity was also affected by the sorts of amines. Although the exact reaction mechanism in this method is not clear at present, the difference in the CL intensity between YOH and NAR may be presumed to be caused by some variables, *i.e.* chemical structures, ionization potentials, state of protonation, pH and *S/N* ratio.

The reversed-phase separation of YOH in serum samples using a TSKgel ODS-80Ts column under acidic condition required an organic solvent with moderate elution power. The optimal content of methanol in the

Fig. 2 Effect of the mobile-phase pH on the CL intensity, as examined by flow injection analysis. Sample concentration: YOH, 500 ng/ml; NAR, 250 ng/ml.

mobile phase was found to be 45%, providing resolution of YOH from endogenous substances in serum. An ion-pairing reagent, sodium 1-heptanesulfonate, was added to the mobile phase to increase the k' values. Previous HPLC methods for the determination of YOH⁴⁻⁸ were based on a solvent extraction of the alkaloid from biological samples; however, such procedures are tedious and time-consuming. Although a solid-phase extraction (SPE) method was already reported by Akbari⁵, nobody reported on the SPE method for human serum. Therefore, we have attempted to establish a simple SPE method of YOH in human serum using a Bond-Elut Certify disposable column. Our developed method resulted in better recoveries of YOH: namely, $97.2 \pm 4.4\%$, $97.3 \pm 2.7\%$ and $98.5 \pm 1.2\%$, (mean $\pm$ SD, $n=5$) at 50, 500 and 1000 ng/ml, respectively (Table 1). The calibration graph for YOH in human serum was linear in the range 50 – 1000 ng/ml ($r=0.9990$). The detection limit of YOH was 30 ng/ml in serum (signal-to-noise ratio=3). The relative standard deviation (RSD) was less than 4.5% at 50 – 1000 ng/ml. Typical chromatograms are shown in Fig. 3.

The time course of the YOH concentration in serum samples from two healthy volunteers, after oral administration of 12 mg of YOH, was traced by the present method (Fig. 4). The pharmacokinetic parameters obtained from the two volunteers are summarized in Table 2. The $t_{\max}$ of YOH was 0.5 h, and $C_{\max}$ was 74 – 94 ng/ml. The half-life value ($t_{1/2}$) of YOH was 6.2 – 6.4 h. The AUC (0 – 4 h) value was 200 – 215 ng h ml⁻¹. The $t_{\max}$ of YOH in the present study was similar, although the intake doses were different from those reported in references.^{3,7}

In conclusion, the combination of HPLC with the CL detection system using the ruthenium complex and solid-phase extraction by the Bond-Elut Certify disposable column was shown to be successfully applicable to the determination of YOH in serum, without any interference from other coexisting substances. Although the present CL method is about 7-times inferior to our pre-

vious fluorescence method⁶ in sensitivity, its sensitivity will be sufficient for pharmacokinetic studies. Several advantages of the CL method are as follows: (1) Many samples can be processed at the same time, and the operation time can be shortened. (2) Due to higher selectivity, better reproducibility and resolution are obtained owing to less interference by other substances in the serum. This method would be useful for the

Fig. 3 Typical chromatograms of (A) blank serum, (B) spiked serum with YOH (250 ng/ml) and NAR (I.S.) and (C) serum after oral administration of YOH (12 mg). Peak: 1, NAR (16.58 min); 2, YOH (21.42 min).

Fig. 4 Serum concentration-time profiles of YOH after single 12 mg oral dose of YOH to two healthy volunteers. Symbol: open, subject A; closed, subject B.

Table 1 Recovery of YOH added to human blank serum ($n=5$)

Added/ng ml ⁻¹	Found/ng ml ⁻¹ (mean $\pm$ SD)	Recovery,% (mean $\pm$ SD)	RSD, %
50	48.7 ± 2.3	97.2 ± 4.4	4.5
500	486.7 ± 13.5	97.3 ± 2.7	2.8
1000	985.2 ± 12.0	98.5 ± 1.2	1.2

Table 2 Pharmacokinetic parameters of YOH after oral dose of 12 mg YOH to two healthy volunteers

Subject	Age/ years	Weight/ kg	$C_{\max}$ / ng ml ⁻¹	AUC(0→4)/ ng h ml ⁻¹	K_{el} / h ⁻¹	$t_{1/2el}$ / h
A	24	65	94	215	0.109	6.4
B	28	70	74	200	0.112	6.2

analysis of other biological samples with a complicated matrix.

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