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Note

Determination of a new dihydropyridine derivative, methyl 3-cyclopentyl-4,7-dihydro-1,6-dimethyl-4-(3-nitrophenyl)pyrazolo[3,4-*b*]pyridine-5-carboxylate, in human serum by high-performance liquid chromatography with electrochemical detection and column switching

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Since the discovery of nifedipine [1], the calcium entry-blocking agent with vasodilatory activity, many analogues of this 1,4-dihydropyridine have been synthesized to improve the therapeutic efficacy for hypertension and cerebrovascular disease [2—4]. Methyl 3-cyclopentyl-4,7-dihydro-1,6-dimethyl-4-(3-nitrophenyl)pyrazolo[3,4-*b*]pyridine-5-carboxylate (I, Fig. 1) is a new potential 1,4-dihydropyridine drug discovered by Shionogi [5] for the next generation of calcium antagonists. The drug is presently being subjected to animal experiments.

Several methods have been proposed for the determination of 1,4-dihydropyridine derivatives such as nifedipine in biological fluids: fluorimetry [6], gas chromatography [7, 8], gas chromatography—mass spectrometry [9], radioreceptor assay [10], reversed-phase liquid chromatography with ultraviolet detection [11, 12] or with reductive-mode electrochemical detection [13]. These methods require time-consuming clean-up procedures or expensive equipment and lack sensitivity or selectivity. Although 1,4-dihydropyridine deriva-

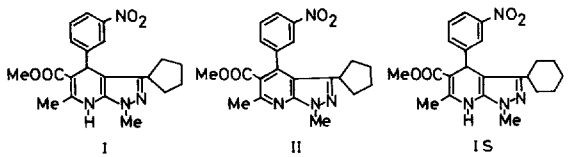

Fig. 1. Chemical structures of compounds I and II and the internal standard (I.S.).

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tives are known to be easily oxidized, oxidative-mode electrochemical detection of these compounds has not been reported until quite recently [14].

We describe here a highly sensitive and rapid method for determining compound I by reversed-phase high-performance liquid chromatography (HPLC) with oxidative-mode electrochemical detection and a column-switching technique to simplify sample pre-treatment and shorten the analysis time.

EXPERIMENTAL

Chemicals

Methyl 3-cyclopentyl-4,7-dihydro-1,6-dimethyl-4-(3-nitrophenyl)pyrazolo-[3,4-*b*]pyridine-5-carboxylate (I), methyl 3-cyclopentyl-1,6-dimethyl-4-(3-nitrophenyl)pyrazolo[3,4-*b*]pyridine-5-carboxylate (II) and methyl 3-cyclohexyl-4,7-dihydro-1,6-dimethyl-4-(3-nitrophenyl)pyrazolo[3,4-*b*]pyridine-5-carboxylate (internal standard, I.S.) were synthesized by Shionogi [5].

Methanol and acetonitrile were of HPLC grade (Merck, Darmstadt, F.R.G.). Water was deionized and distilled before use. Ion-pair reagent, sodium 1-octanesulphonate (PIC B-8, Waters Assoc., Milford, MA, U.S.A.), was dissolved in water (1 bottle per l) before use. All other chemcials and solvents were of analytical reagent grade.

Chromatographic and cyclic voltammetric system

The column-switching chromatographic system consisted of a Model L-5000 solvent delivery pump (Yanagimoto, Kyoto, Japan), a Model 7125 syringe-loading sample injector with a 100-μl sample loop (Rheodyne, Berkeley, CA, U.S.A.), a Model LC-4B electrochemical detector (Bioanalytical Systems, West Lafayette, IN, U.S.A.), a Model A-60-S sub-pump (Eldex, Menlo Park, CA, U.S.A.), a Model PD-3000 pulse damper (Chemco Scientific, Osaka, Japan) and a Model 7000A six-port switching valve (Rheodyne).

The electrochemical detector consisted of a Model TL-5 thin-layer detector cell with a glassy carbon working electrode and an Ag/AgCl reference electrode.

Concentrations were calculated using a Model C-R2AX integrator (Shimadzu, Kyoto, Japan). The column-switching system was controlled with a Model FA-1 programmable controller (Izumi, Osaka, Japan).

Separations were performed on two octadecylsilane columns; one was a Nucleosil C_{18} pre-column (10 μm, 5 cm × 4.0 mm I.D., home-packing, Macherey-Nagel, Düren, F.R.G.) and the other was a Nova-Pak C_{18} analytical column (5 μm, 15 cm × 3.9 mm I.D., Waters). A Spheri-5 RP-18 OD-GU column (5 μm, 3 cm × 4.6 mm I.D., Brownlee Labs. Santa Clara, CA, U.S.A.) was used to guard the first two columns.

A Model VMA-010 cyclic voltammetric analyser (Yanagimoto) was used for cyclic voltammetric measurements, and a Model WX-1000 x—y recorder (Graphtec, Tokyo, Japan) was used to record the cyclic i versus E curves. A glassy carbon disk of 3 mm diameter was used as a working electrode. The surface of the disk was polished to a mirror finish with alumina powder (0.05 μm) on an acrylic resin plate before use. An Ag/AgCl electrode and a platinum wire were used as a reference and an auxiliary electrode, respectively.

Cyclic voltammetry

Cyclic voltammetry was performed at a concentration of ca. 2 m*M* I or II

in 70% methanol containing 0.05 *M* sodium perchlorate. The solution was stored in a 15-ml tube and covered with a PTFE cap through which the three electrodes and a nitrogen purge tube were inserted. After 15 min of bubbling with nitrogen, the flow was stopped and the cyclic voltammogram of the stationary solution was obtained at room temperature.

Chromatographic conditions

Chromatography was performed at room temperature. A mixed solution of methanol—acetonitrile—0.005 *M* sodium 1-octanesulphonate (8:5:8) was used as a mobile phase at a constant flow-rate of 1.3 ml/min. The mobile phase was filtered with a Type FR-40 membrane filter (0.4 μm, Fuji Photo Film, Tokyo, Japan) and degassed under reduced pressure before use.

The potential of the electrochemical detector was set at +0.80 V vs. Ag/AgCl.

The solvent for washing the pre-column was of the same composition as the above mobile phase and was delivered at a flow-rate of 3.0 ml/min.

Choice of internal standard

The internal standard (I.S.) was chosen because of its structural similarity to compound I: the I.S. and compound I were found to have physicochemically similar properties, such as electrochemical response, extraction efficiency and chromatographic retention.

Procedure

To a 12-ml brown centrifuge tube, 100 μl of 50 ng/ml I.S. in methanol were added and evaporated to dryness under reduced pressure, followed by the addition of 1 ml of human serum containing 0.5—20 ng/ml I and shaking for 0.5 min. To the mixture, 1 ml of water and 5 ml of ethyl acetate were added. After the mixture had been shaken for 10 min with a mechanical shaker, it was centrifuged (1100 *g*, 10 min). To a clean 10-ml brown tube, 4 ml of organic layer were transferred and evaporated to dryness under reduced pressure. The residue was reconstituted in 80 μl of the mobile phase and 50-μl portions were injected into the chromatographic system (prior to chromatography, the sample solution was stored at 0°C in the dark).

Precision and linearity

Within-day and day-to-day precision and linearity were determined and calculated by peak-area ratios (I/I.S.). To assess within-day precision, human serum samples containing 20 or 1 ng/ml I were assayed six and five times in a day, respectively. To assess day-to-day precision, human serum samples spiked with 1 ng/ml I were assayed five times per day for three days.

RESULTS AND DISCUSSION

Cyclic voltammetry

Cyclic voltammograms of compound I and its electrochemically oxidized product II are shown in Fig. 2. The cathodic peaks of compound I were observed at —0.90 V (Ra) and —0.59 V (Rb_1), and that of compound II was ob-

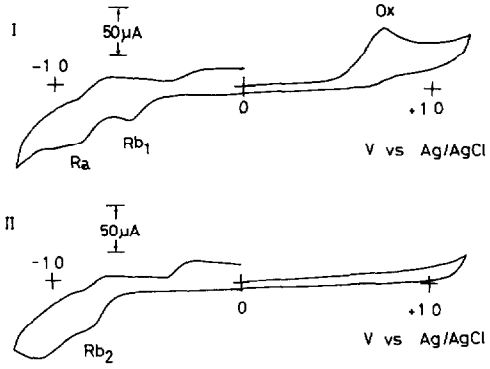

Fig. 2. Cyclic voltammograms of 2 m*M* I (upper) and a saturated solution of compound II (lower) in 70% methanol containing 0.05 *M* sodium perchlorate. Scan-rate, 200 mV/s; working electrode, glassy carbon.

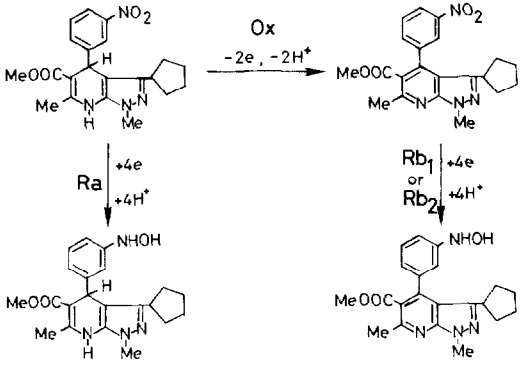

Fig. 3. Proposed mechanism for the redox of compound I. Ox, Ra, Rb_1 and Rb_2 correspond to the cyclic voltammogram peaks of Fig. 2.

served at −0.80 V (Rb_2). On the other hand, the anodic peak of compound I was obtained at +0.75 V (Ox), but that of compound II was not.

Fig. 3 shows the proposed mechanism for electrochemical oxidation and reduction (redox) of compound I and its products, and shows consistency with the cyclic voltammograms of Fig. 2. Peak Ra corresponds to the four-electron reduction of the nitro group to hydroxylamine. Peak Ox corresponds to two-electron oxidation of the dihydropyridine form to the pyridine form (II). Peaks Rb_1 and Rb_2, which are essentially the same, correspond to four-electron reduction of the nitro group of the pyridine form to the hydroxylamine group. This mechanism is basically the same as that of nifedipine proposed by Bratin and Kissinger [13].

Consideration of the redox properties shows that compound I can be determined by either reductive- or oxidative-mode electrochemical detection. We selected the oxidative mode, because the cathodic peak potential of compound I, −0.90 V, is high and reductive-mode electrochemical detection has some problems, such as interference by oxygen.

Chromatographic system with column switching

The chromatographic system with just an analytical column could not

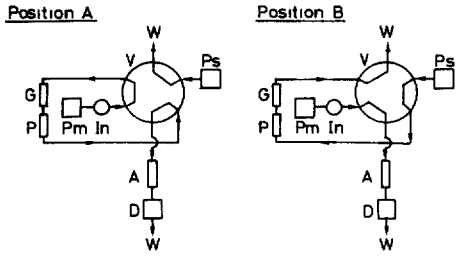

Fig. 4. Schematic diagrams of the chromatographic system with column switching. Position A = injection and pre-separation mode; position B = analysis and back-flushing mode; Pm = main pump; Ps = sub-pump; In = injector; V = six-port switching valve; D = detector; G = guard column; P = pre-column; A = analytical column; W = to waste.

eliminate late-eluting peaks during 60—120 min. Thus, a column-switching technique was designed to remove the late-eluting peaks. A schematic diagram of this system is shown in Fig. 4.

When a sample is injected, the column-switching valve is set to position A. After the compounds of interest, I and I.S., are eluted to the analytical column from the pre-column, the valve is switched to position B. When the valve is at position B, the guard column and the pre-column are back-flushed with an eluent using a sub-pump, and the analytical column is used to separate compound I and I.S. from the other interferences. The eluent delivered by the sub-pump is of the same composition as the mobile phase, to prevent baseline drift. After all the peaks of interest are detected, the valve is switched back to

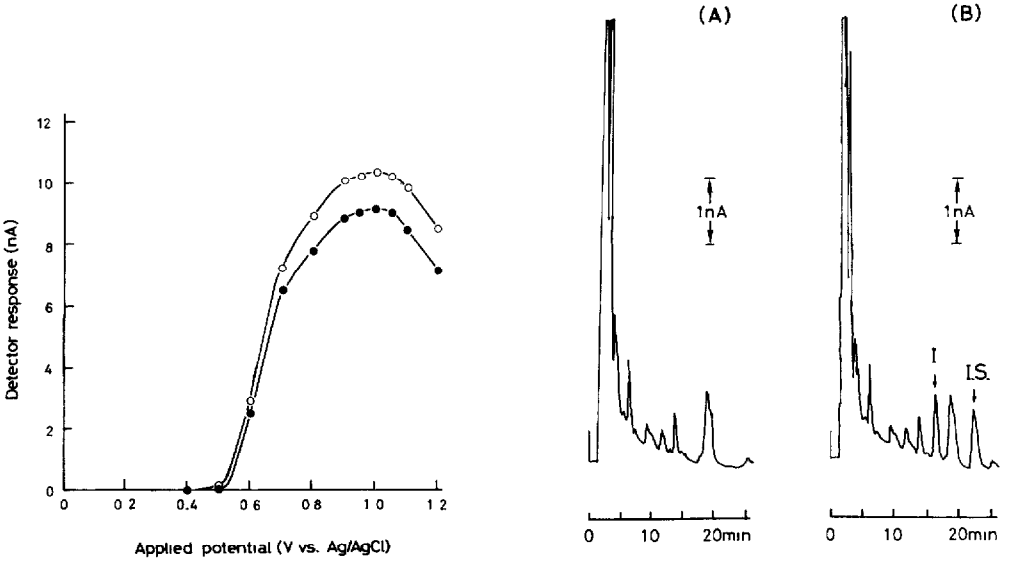

Fig. 5. Relationship between detector response and applied potential for 200 pmol of compound I (○) and I.S. (●). Mobile phase, methanol—acetonitrile—0.005 *M* sodium 1-octanesulphonate (8:5:8); flow-rate, 1.3 ml/min; electrode, glassy carbon.

Fig. 6. Chromatograms of drug-free human serum blank (A) and human serum spiked with 20 ng/ml I and 20 ng/ml I.S. Detector potential, +0.80 V vs. Ag/AgCl; injection volume, 50 μl; other conditions as in Fig. 5.

position A. The entire cycle is started again at position A, when the next sample is injected.

This method shortens the analysis time to ca. 25 min per sample.

Electrochemical detection

Fig. 5 shows the hydrodynamic voltammograms of compound I and I.S. in the potential range of +0.40 V to +1.20 V. The voltammograms demonstrate the similarity of voltammetric behaviour of the two dihydropyridines: both show only one oxidative wave having a peak at +1.00 V. In general, lower applied potential allows higher selectivity and reduces the noise. The choice of a detector potential of +0.80 V seems to be a good compromise between an adequate current response to dihydropyridines and low background current.

Typical chromatograms of blank human serum (A) and human serum spiked with 20 ng/ml I and 20 ng/ml I.S. (B) are shown in Fig. 6.

The relative standard deviations for the within-day assay were 2.4% at 20 ng/ml I (n = 6) and 11.2% at 1 ng/ml (n = 5), and that for the day-to-day assay was 13.2% at 1 ng/ml (n = 15). A good linear relationship was found between the peak-area ratio and the concentration of compound I in human serum in the range of 0.5–20 ng/ml. The minimum detection limit was 0.3 ng/ml (signal-to-noise ratio = 2).

CONCLUSION

A simple, rapid, highly sensitive and selective method was developed for the determination of compound I in human serum by the combined use of electrochemical detection and a column-switching technique. Our results suggest that the method is useful for pharmacokinetic study of compound I and can be applied to assays of other dihydropyridine derivatives.

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