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Sensitive determination of the phosphodiesterase III/IV inhibitor zardaverine in human serum by direct sample injection, automated precolumn clean-up and high-performance liquid chromatography

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ABSTRACT

A high-performance liquid chromatographic method is described for the determination of the phosphodiesterase III/IV inhibitor zardaverine in serum by using fully automated clean-up of large-volume serum samples on a semi-preparative-scale precolumn followed by chromatography on two analytical columns operated with two different solvent systems. The switching of the analytical columns provides the necessary specificity and sufficient sensitivity for UV detection is obtained by the sample volume. The method was shown to give nearly quantitative recovery, allowing the use of external standard quantification. Good precision and linearity within the concentration range 1–50 ng/ml could be demonstrated. The method is suitable for routine measurements in support of kinetic studies of zardaverine in man.

INTRODUCTION

Zardaverine [6-(4-difluormethoxy-3-methoxyphenyl)-3(2*H*)-pyridazinone] is a new phosphodiesterase (PDE) III/IV inhibitor (Fig. 1), exhibiting bronchospasmolytic and anti-inflammatory activity as shown both *in vitro* and in animal models [1,2]. To

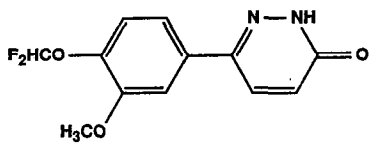

Fig. 1. Structure of zardaverine.

establish its potential therapeutic use for treatment of asthma in man, zardaverine is currently being evaluated in clinical phase II. In order to support human kinetic studies, a sensitive method had to be developed for the determination of the drug in serum down to the low ng/ml range.

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EXPERIMENTAL

Materials

Hypersil ODS (5 μm), LiChroprep RP-2 (25–40 μm), empty precolumns (30 × 8 mm I.D., Hyperchrom) and empty analytical columns (125 × 4.6 mm I.D., Hyperchrom) were supplied by Grom (Amerbuch, Germany). The internal length of the precolumn was shortened to 10 mm by insertion of a 20 × 8 mm PTFE rod with a bore of 0.25 mm. All column endings were closed by metal sieves rather than by frits.

Zardaverine and the marker compound [6-(3,4-methylenedioxyphenyl)-3(2*H*)-pyridazinone] were synthesized at Byk Gulden (Konstanz, Germany) and were >99% pure as tested by high-performance liquid chromatography (HPLC). Methanol and acetonitrile were obtained from E. Merck (Darmstadt, Germany) and were of LiChrosolv quality. Reagents were purchased from E. Merck and were of analytical-reagent grade, unless stated otherwise.

Apparatus

The first part of the HPLC system consisted of a Gynkotek (Munich, Germany) Model 300 high-precision pump. The auxiliary pump was a Gilson Model 302 (Abimed, Langenfeld, Germany), which was connected to a Gilson Model 231-401 auto-sampling injector (Abimed), the latter consisting of a sample controller, a Model 231 sample injector and a Model 401 diluter. Column switching of the precolumn to the first analytical column was done by means of a pneumatic Rheodyne Model 7010A six-port valve (Latek, Heidelberg, Germany), which was controlled by an external contact of the sample controller. A valve of the same type was used for the transfer of the heart-cut from the first analytical column to the second chromatographic system, which consisted of a Hewlett-Packard (Waldbronn, Germany) Model HP1084B liquid chromatograph. The second switching valve was contributed by the column switching option 79823A of the HP1084B system. The effluents from the two analytical columns were monitored at 253 nm by two Kratos (Karlsruhe, Germany) Spectroflow 757 UV detectors and the signals were recorded on a Hitachi Model D-2000 chromato-integrator (E. Merck) for the first detector and the HP79850B integrator of the HP1084B for the second detector. Integration of chromatograms was performed by means of a Hewlett-Packard HP 3357 laboratory data system.

The analytical columns were packed by using an HPLC column packing unit (Latek, Heidelberg, Germany). Precolumns were dry-filled.

Columns, eluents and column switching

The precolumn was filled with LiChroprep RP-2 (25–40 μm). Both analytical columns contained Hypersil ODS (5 μm). The serum samples were transfered to the precolumn by a stream of 10 m*M* aqueous ammonium phosphate (PH 7) at a flow-rate of 2.0 ml/min. After a flushing period of 10 min to remove matrix components, the precolumn was connected with the first analytical column via the switching valve for 2 min, after which the columns were disconnected again.

The mobile phases for the analytical columns consisted of a mixture of 10 m*M* aqueous ammonium phosphate (pH 7) and either methanol [60:40 (v/v), column 1] or acetonitrile [75:25 (v/v), column 2]. The flow-rate for both analytical columns was set at 1.0 ml/min and both were operated at 40°C. The heart-cut from the first analytical column extended from 17 min until 20 min after injection.

Preparation of aqueous stock solutions and serum external standards

A 10-mg amount of zardaverine or the marker compound was dissolved in 1 ml of 1 *M* sodium hydroxide and 3 ml of methanol by warming. The solutions were made up to 100 ml (zardaverine) or 10 ml (marker compound) with doubly distilled water to provide the stock solutions. The stock solution of the marker compound was further diluted with water to a concentration of 5 μg/ml. For the preparation of spiked serum standards, each 1.3 ml of human serum was mixed with an equal volume of the appropriately diluted aqueous stock solution of zardaverine and 20 μl of the diluted solution of the marker compound were added, the concentration of the marker thus being 77 ng/ml.

Serum samples (1.3 ml) from kinetic studies were diluted 1:1 with water and, after addition of 20 μl of the solution of the marker compound, a total of 2000 μl of the diluted sample was injected.

Precision, day-to-day variation, accuracy and recovery

The precision of the assay was tested by tenfold injections of spiked serum standards at concentrations of 2, 20 and 50 ng/ml. These data were also used to calculate the accuracy as the percentage difference between the expected and the measured values. The day-to-day variation was calculated from the peak areas of spiked serum standards at concentrations of 5, 20 and 50 ng/ml. One batch of spiked standards was produced at each concentration and frozen at −20°C as aliquots, which subsequently were thawed and analysed ($n = 10$ per concentration) within several kinetic experiments over a period of 16 weeks.

The recovery of zardaverine on the precolumn was determined by comparison of the mean peak areas after injection of spiked serum standards on the precolumn with the mean peak areas after direct injection of zardaverine dissolved in eluent 2 on the second analytical column. This procedure ensures that potential losses in performing the heart-cutting are also considered. The recovery was determined at concentrations of 2, 20 and 50 ng/ml after fourfold injections.

Calibration

The calibration line for zardaverine was constructed from the mean (± S.D.) peak areas obtained after fourfold injection of spiked serum standards at concentrations of 1, 2, 5, 10, 35 and 50 ng/ml.

RESULTS AND DISCUSSION

Zardaverine was determined by direct injection of serum onto a precolumn with fully automated sample clean-up in combination with a column-switching method [3,4]. As the serum concentrations of zardaverine in man are in the low ng/ml range, the specificity and sensitivity of the HPLC assay had to be enhanced by transfering a heart-cut from the first analytical column to a second column (Fig. 2). The gain in selectivity on the second column was achieved by changing the organic modifier from acetonitrile to methanol while keeping the stationary phase on both columns the same. The heart-cut window (width 3 min) was checked for each chromatogram by means of the marker compound, which is eluted from the first analytical column 1 min before the start of the heart-cutting.

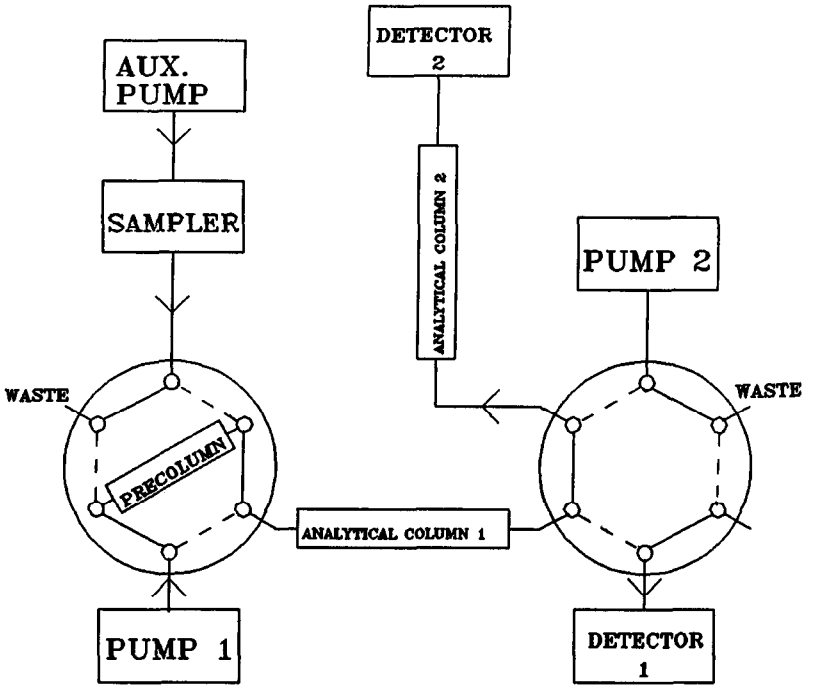

Fig. 2. Scheme of column switching.

The sensitivity required for the low concentrations of zardaverine was achieved by injecting 2 ml of 1:1 diluted serum onto the precolumn. In order to compensate for the heavy sample load, an I.D. of 8 mm was chosen for the precolumn, which thus allowed up to 35 serum samples (= 35 ml) to be processed before replacement of the precolumn was considered necessary. In order to increase the lifetime of the first analytical column, the precolumn was thoroughly flushed for 10 min after sample injection.

The connection time of the precolumn to the first analytical column was optimized by stepwise reduction until the peak area of zardaverine started to decrease. An optimum, yet safe, connection time was found to be 3 min. Optimization of the connection time was essential as a gradual pressure build-up on the precolumn tended to cause a slight decrease in the flow-rate of pump 1 with a consequent shift of the zardaverine peak away from the centre of the heart-cut window. A short connection time of the precolumn obviously would limit this effect. The cycle time for one analysis could be restricted to 32 min, the zardaverine peak eluting at 28 min.

Fig. 3 shows chromatograms of a blank serum sample (B), a serum sample spiked with 20 ng/ml of zardaverine (C) and a serum sample obtained from a volunteer 0.25 h after oral administration of 6 mg of zardaverine (D). These chromatograms were recorded by detector 2 on the second analytical column. Chromatogram A shows the signal of detector 1, the heart-cut region appearing as a plateau, as the detector is bypassed within this time period. Also visible in this plot is the peak of the marker compound.

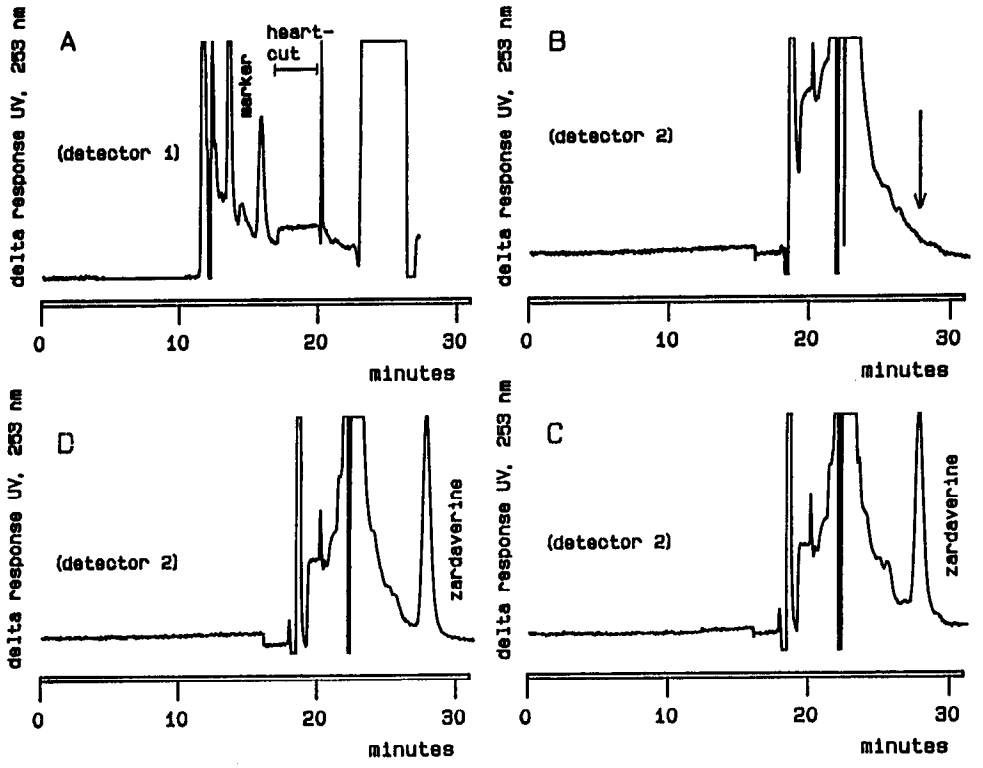

Fig. 3. HPLC after injection of serum samples. Injection volume, 2 ml. (A) Spiked serum standard, trace from detector 1 showing the heart-cut region and the marker compound; (B) blank serum; (C) serum spiked with 20 ng/ml of zardaverine; (D) serum from a human volunteer 0.25 h after oral administration of 6 mg of zardaverine.

Linearity of the assay was tested from 1 to 50 ng/ml, the limit of quantification thus being 1 ng/ml. The regression line obtained from the linearity plot (28 measurements) obeyed the equation $y = 1.04x - 0.84$, the correlation coefficient being 0.9995. The accuracy was between −20% and 0.6% for the concentration range between 1–50 ng/ml (Table I). The relative standard deviation (R.S.D., $n = 10$) for the within-day precision at 2, 20 and 50 ng/ml were 11.8, 2.1 and 1.1%, respectively. The

TABLE I

PRECISION (R.S.D.), ACCURACY AND RECOVERY OF THE ZARDAVERINE ASSAY

Mean concentration ($n = 10$) (ng/ml)		R.S.D. (%)		Accuracy (%)	Recovery (%) ($n = 4$)
Taken	Found	Within-day	Day-to-day		
2	1.6	11.8	–	−20.0	83.6
5	–	–	13.7	–	–
20	19.7	2.1	4.7	− 1.5	98.3
50	52.0	1.2	4.3	− 4.0	101.2

R.S.D.s for the day-to-day variation at concentrations of 5, 20 and 50 ng/ml over a period of 16 weeks were 13.7, 4.7 and 4.3%, respectively. As the peak areas of zardaverine (all standards being from the same batch) did not decrease noticeably, zardaverine in serum is stable at $-20°C$ over this time period. The recoveries at 2, 20 and 50 ng/ml were 84, 98 and 101%, respectively, and may thus be considered to be quantitative, allowing the use of the external standard method for quantification.

CONCLUSIONS

Using automated precolumn clean-up, followed by chromatography on two analytical columns, the HPLC assay allows the rapid, sensitive and accurate determination of zardaverine in serum at concentrations down to 1 ng/ml without any sample pretreatment except for the addition of a marker compound to the serum sample and a 1:1 dilution step with water. The method is suitable for routine measurement, in particular for the characterization of the kinetics of the drug in man.

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