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Note

Analysis of Zaprinast in rat and human plasma by automated solid-phase extraction and reversed-phase high-performance liquid chromatography

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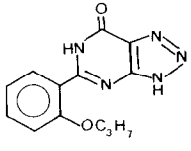
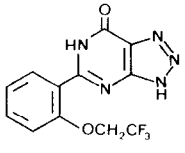
Zaprinast, 1,4-dihydro-5-(2-propoxyphenyl)-1,2,3-triazolo[4,5-*d*]pyrimidin-7-one (Fig. 1a), is a selective inhibitor of phosphodiesterase (PDE) isoen-



(a) Zaprinast



(b) Internal Standard

Fig. 1. Structures of Zaprinast (a) and internal standard (b).

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zymes. Inhibition of these enzymes results in elevated levels of cGMP, accompanied by smooth muscle relaxation. It has potential use in the treatment of conditions which respond to vasodilation, such as congestive heart failure, angina and hypertension. As a bronchodilator it has demonstrated anti-asthmatic properties [1]. It has also been shown to relax gastrointestinal smooth muscle [2], and delay gastric emptying [3] and so is of interest for the treatment of lesions of the gastro-intestinal tract.

In order to follow the kinetics of the drug in small animals a novel analytical method was required. The aim was to produce an automated assay that was accurate, precise and suitable for use with small sample volumes (less than 50 μl). The advantages of liquid–solid extractions in this conjunction have been discussed elsewhere [4,5]. In this assay an Analytichem AASP (Advanced Automated Sample Processor) has been linked to a Gilson 222/401 liquid-handling station to produce a fully automated process, eliminating manual sample extraction.

EXPERIMENTAL

Chemicals and reagents

Ammonium acetate (AnalaR grade), glacial acetic acid (Pronalys), methanol (AnalaR) and acetonitrile (HPLC grade) were supplied by May and Baker (Dagenham, U.K.). Sodium hydroxide (AnalaR grade) was supplied by BDH (Poole, U.K.). Filtered, HPLC-grade water was obtained daily from a Milli-Q water purification system [Millipore (U.K.), Harrow, U.K.]. Zaprinast and the internal standard for the assay, 5-[2-(2,2,2-trifluoroethoxy)-phenyl]triazolo[4,5-*d*]pyrimidin-7-one, were supplied by Dr. W.J. Coates, Medicinal Chemistry Department, SK&F Research (The Frythe, U.K.). Structures of both compounds are shown in Fig. 1. Stock solutions of Zaprinast and internal standard (both 100 μg ml^{-1}) were prepared in methanol and were stable when stored for up to one month at 4 °C. Spiking solutions were prepared depending on the anticipated concentrations of Zaprinast in the samples. The methanolic stock solution was diluted with water to 4.0 μg ml^{-1} for calibrations in the range 1.0–25.0 μg ml^{-1}. For calibrations from 5.0 to 50.0 μg ml^{-1}, the Zaprinast stock solution was diluted 1:10 to 10.0 μg ml^{-1}. Spiking solutions of internal standard (0.4 or 1.0 μg ml^{-1} depending on the calibration range) were prepared in 0.1 *M* acetic acid, when required.

Collection and storage of samples

Blood was drawn into tubes containing lithium heparin as anticoagulant. The plasma was separated by centrifugation at 1500 *g* for 10 min, transferred to non-heparinised tubes and stored at −20 °C. All samples were assayed within one week of sample receipt, due to insufficient information on the stability of Zaprinast on storage.

Apparatus

The chromatograph consisted of a Waters Assoc. Model 590 pump [Millipore (U.K.)], a Kratos Spectroflow 783 or 757 detector (Severn Analytical, Gloucester, U.K.) monitoring at 275 nm and an AASP, manufactured by Analytichem (Harbor City, CA, U.S.A.) and supplied by Jones Chromatography (Llanbradach, U.K.). AASP solid-phase extraction cassettes were also supplied by Jones Chromatography. Automated sample preparation was performed by a Gilson 222 autosampler and 401 dilutor (Anachem, Luton, U.K.), linked directly to the AASP. Chromatographic recordings and integrations were performed on an LDC 301 recording integrator (Laboratory Data Control, Stone, U.K.). The separation was carried out on a 15 cm × 4.6 mm I.D. column, prepacked with Altex Ultrasphere^{TM} C_8, 5 μm particle size (supplied by Beckman-RIIC, High Wycombe, U.K.), maintained at 40°C using a Dupont 8800 column oven. A mobile phase of acetonitrile–0.05 *M* acetate buffer adjusted to pH 4.0 (35:65, v/v) was used, with a flow-rate of 1 ml min^{-1}. Any dissolved gases were removed by sparging with helium before and during use.

Automated extraction

The combination of Gilson 222 autosampler and AASP enabled sample extraction to be performed just prior to injection. A Gilson 401 dilutor, in conjunction with the 222, was used to draw the liquids required for solid-phase sample extraction into a holding loop in reverse order. The liquid 'train' was then passed through a C_2 AASP cartridge in situ in the AASP. After sample extraction, the mobile phase was switched through the cartridge, eluting Zaprinast and internal standard directly into the analytical column. This process is summarised in Fig. 2.

Three programs were required for the assay of Zaprinast in plasma. Program

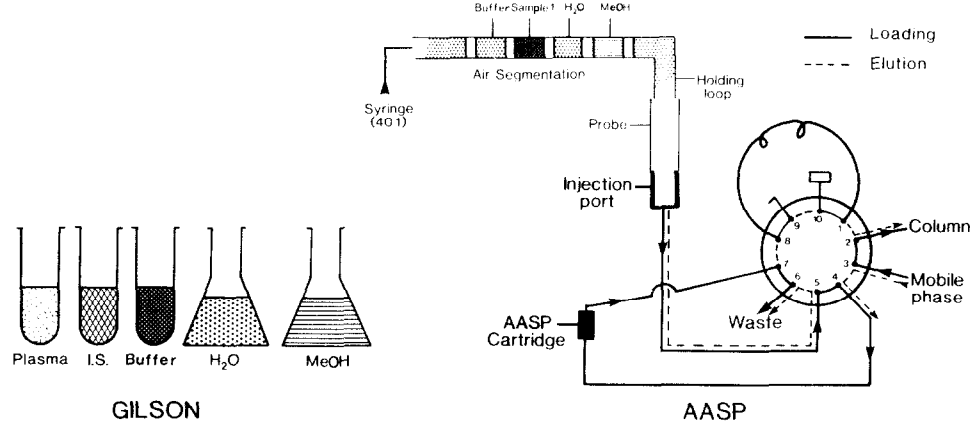

Fig. 2. Diagram of the Gilson–AASP system.

PURGE was used to flush the Gilson–AASP prior to and after use. Program SAMPLE was used to analyse samples. If it was necessary to repeat any analyses, program REASSAY was used. Further details on these programs may be obtained from the authors' upon application.

The following procedure is followed for sample analysis. Four reagent bottles containing 0.1 *M* acetic acid plus internal standard, pH 8 acetate wash (0.1 *M*), water and methanol are placed in the Gilson 222 bottle rack. In addition, water is placed in the autosampler's main solvent reservoir. A blank cassette is placed in the AASP and the software program PURGE run on the Gilson 222 prior to any sample extraction. The software program SAMPLE is then run for sample analysis. The 401 diluter in conjunction with the Gilson 222 first draws up 1000 μl of the internal standard stock solution in 0.1 *M* acetic acid, followed by 35 μl of air. This is dispensed into sample No. 1 using the probe. The diluted sample is mixed by drawing 1000 μl air into the probe, then bubbling this back through the liquid. The solutions used in solvent extraction are then drawn into the holding loop. The final wash stage of the extraction is drawn into the holding loop first, i.e. 300 μl of pH 8 acetate wash (0.1 *M*). This is followed by 400 μl of sample diluted with internal standard, 1 ml of water and finally 1 ml of methanol. The solutions are kept separate from each other, by segmentation using 35-μl aliquots of air. Once sample and wash stages have been loaded into the holding loop, the probe moves to the injection port. The train of liquid in the holding loop is then slowly pushed through the C_2 AASP cartridge and out to waste. The total volume passing through the cartridge includes an additional 625 μl of the water from the main water reservoir, following the sample train. This ensures that all wash phases have fully passed through the cartridge, and out of the valve to waste, before injection. On command from the Gilson 222, the Valco valve on the AASP is switched to inject mode and mobile phase is directed through the cartridge, eluting the analyte onto the analytical column. The integrator run commences simultaneously. At a preset time (0.6 min) the AASP valve is reset to the load position. The second AASP cartridge is brought in line and the next sample loaded as before. The high-performance liquid chromatographic (HPLC) run for the first sample continues to the end of the integrator analysis time (8 min). Following a short delay for print-out from the integrator, the valve switches again to commence the HPLC analysis for the second sample. This sequence of events continues until all the plasma samples in the rack have been processed.

Re-extraction of samples in the sample rack, which have already been diluted with acid and internal standard, can be performed using the REASSAY program. This program will repeat the extraction sequence, omitting the addition of acid and internal standard. The mixture of plasma, acetic acid and internal standard can be left for up to 24 h before re-extraction, with no adverse effect.

Sample preparation prior to automation

The initial preparation of samples, and spiking of standards or quality controls, was performed manually. Calibration standards were prepared in the ranges 1.0–25.0 and 5.0–50.0 $\mu g\ ml^{-1}$ by addition of 5–125 μl of Zaprinast spiking solution to drug-free plasma (20 μl). No internal standard was added at this stage, as it was dispensed automatically by the Gilson 222 during sample processing. Standards and plasma samples for assay (20 μl) were made up to constant volume with a methanol–water mixture of the same proportions present in the spiking solution, to the same volume. This was performed in 5-ml plastic tubes, which were then placed in the Gilson 222 sample rack ready for analysis.

RESULTS

Chromatography

Under the conditions described, the retention time of the internal standard was approximately 3.7 min and that for Zaprinast was 5.2 min. These times varied slightly if column or mobile phase were changed, but not so that the analytical results were affected. Typical chromatograms of rat plasma extracts containing Zaprinast and internal standard are shown in Fig. 3. A small peak produced by endogenous material is sometimes observed, of similar retention time to Zaprinast. This peak corresponds to a concentration considerably below the limit of quantification for the assay and does not affect calculated results.

Calibration

Calibration lines were produced by plotting the peak-area ratio of Zaprinast to internal standard against known concentrations of Zaprinast added to drug-free plasma. Two-variable linear regression analysis was then used to construct a calibration line, and concentrations of Zaprinast were calculated by relating the respective peak-area ratio to the line. A typical calibration line constructed for the range 1.0–25.0 $\mu g\ ml^{-1}$ was described by the equation $y = 0.042347x + 0.007238$, where x represents concentration in $\mu g\ ml^{-1}$ and y peak-area ratio of Zaprinast to internal standard. The precision of the method was calculated as the coefficient of variation of replicate assays of plasma standards containing Zaprinast in the two different calibration ranges. Six replicates at high and low concentrations and three replicates of intermediate concentrations were assayed. Calibrations were performed on three different days, so that the coefficients of variation reported include day-to-day variation. The accuracy of the method was assessed by re-fitting the ratios from the standards used to assess precision into the regression equation derived from these data and calculating the bias. Calculated values for the precision of the assay were between 3 and 13% for all concentrations from 1 to 50 $\mu g\ ml^{-1}$. The values

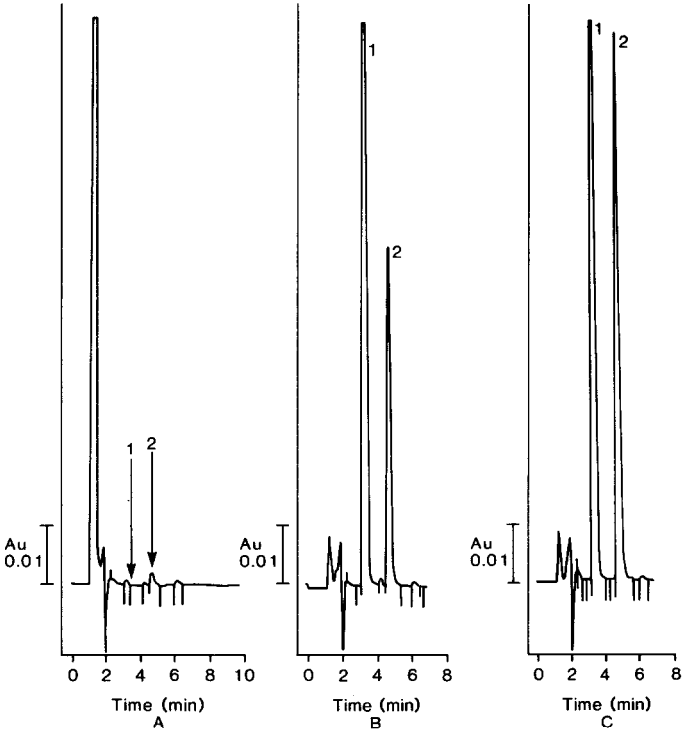

Fig. 3. Typical chromatograms of (A) control rat plasma extract, (B) rat plasma extract spiked with Zaprinast (30 μg ml^{-1}) and internal standard and (C) rat plasma extract following and intravenous infusion of Zaprinast. Peaks: 1 = internal standard; 2 = Zaprinast.

calculated for the bias ranged from 0.4 to 5.8%, except for the 1 μg ml^{-1} standard, where the bias was calculated at 18%.

Recovery

To assess the recoveries of Zaprinast and internal standard from plasma, the HPLC responses of these compounds in extracted standards were compared with the responses of known amounts on column. Recovery of Zaprinast from plasma was assessed at all concentrations used to produce calibration data. Recovery was greater than 88% at all concentrations, the mean recovery being 93%. Recovery of internal standard was assessed at the concentration present in all extracted samples, and mean recovery was calculated to be 103% by the same method.

DISCUSSION

This method demonstrates the advantages of combining solid-phase extraction followed by HPLC with a fully automated sample handling system. It is

just one of many applications of the Gilson–AASP combination used in the authors' laboratory. This system was gained in popularity because of its ease of use and adaptability offered through simply altering the program variables. The use of the system is dependent on the stability of the analyte in plasma or urine at room temperature for a period at least as long as the total run time. Zaprinast has been shown to be stable under these conditions for at least 24 h. The assay of Zaprinast is an example of how this combination is useful for research using small animals, where sequential bleeds of only 50 μl of blood are taken. It also has the potential for greater sensitivity by increasing sample volume.

The method described is easy to use, requires minimal human input and has the potential for high sample throughput. Thus it is ideal for a busy laboratory, freeing the scientist for other duties.

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