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Short Communication

An assay for prizidilol in plasma and urine by high-performance liquid chromatography

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Introduction

Treatment of essential hypertension with drugs which cause vasodilation alone is rarely effective because of the reflex tachycardia which occurs. This can be overcome by the co-administration of a β-adrenoreceptor blocking agent, which will prevent or reduce the cardiac stimulation resulting from the action of the vasodilator [1]. Prizidilol hydrochloride (SK&F 92657-A_2, **I** in Fig. 1) is a new antihypertensive agent which combines both pre-capillary vasodilating and β-blocking activities in one molecule [2].

Anaytical methods were required to follow the kinetics of the drug in man and experimental animals during clinical studies and toxicology. The method reported here involves the reaction of prizidilol with *p*-anisaldehyde to form a hydrazone derivative, followed by extraction and high-performance liquid chromatography (HPLC) with UV detection. Derivatization is necessary because the drug is unstable at alkaline pH and has not been successfully extracted from plasma and urine at acid or neutral pH. The method follows a similar approach to that used for hydralazine [3], and is not specific in that it will determine not only unchanged drug but also its acid-labile metabolites.

Experimental

Materials

All chemicals used in this assay were analytical grade with the following exceptions: *p*-anisaldehyde, di-*n*-butylamine (B.D.H. Poole) and propan-2-ol (Fisons, Loughborough) were reagent grade. Dichloromethane and methanol (Rathburns, Walkerburn) were HPLC grade.

Polypropylene test tubes (12 ml, push cap) were obtained from Henleys Medical Supplies, London. Screw-cap polypropylene tubes (12 ml) were obtained from R. and L. Slaughter Ltd, Romford.

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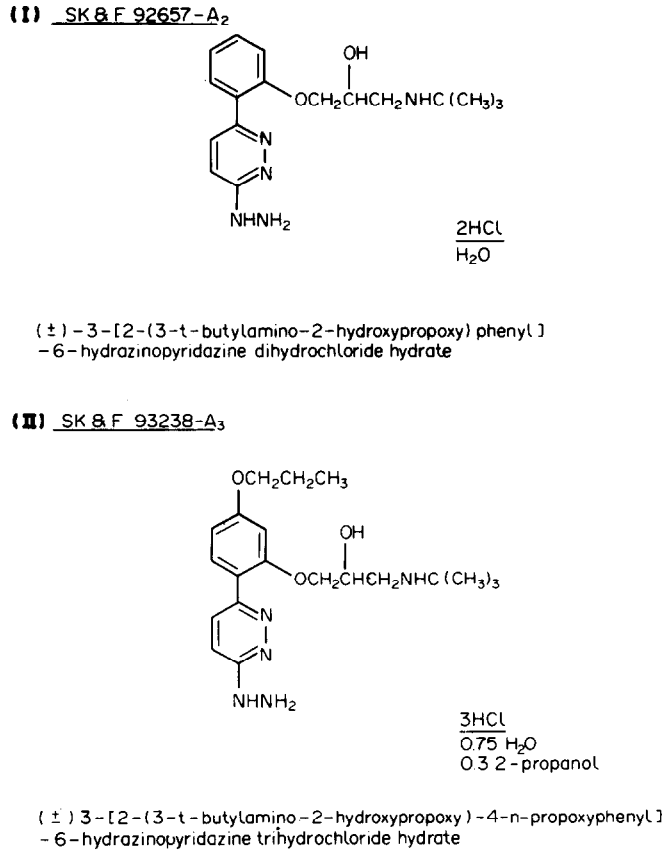

Figure 1
Structure of SK&F 92657-A_2 and SK&F 93238-A_3.

Water was purified by deionization, then distilled in an all-glass apparatus and stored in glass containers. The solution of anisaldehyde (1% v/v in methanol) was prepared fresh on each day of the analysis. All other reagents were stored for up to 1 month at room temperature. SK&F 92657 hydrochloride and the internal standard, SK&F 93238 trihydrochloride (**II** in Fig. 1), were supplied by Medicinal Chemistry (SK&F, Welwyn), and were found by HPLC with UV detection to be 99 and 98% pure respectively. SK&F 92657 anisaldehyde hydrazone and SK&F 93238 anisaldehyde hydrazone were found by HPLC with UV detection to be 99 and 94% pure respectively. The authenticity of the structure of each compound was supported by NMR, mass-spectrometry, IR, UV and micro-analysis. ^{14}C-Prizidilol was synthesized by Radiochemistry (SK&F, Welwyn). It was found to be 99% radiochemically pure by reverse-isotope dilution and had a specific activity of 41.5 μCi/mg. The molecule was labelled in positions 4 and 5 in the pyridazine ring.

Preparation of standard solutions

For the plasma assay, stock solutions of prizidilol and the internal standard, (SK&F 93238 trihydrochloride) were prepared in methanol at concentrations of 10 mg l^{-1} (base equivalent) and 20 mg l^{-1} (base equivalent) respectively.

For the urine assay, stock solutions of prizidilol and the internal standard were prepared in methanol at concentrations of 50 mg l^{-1} (base equivalent) and 30 mg l^{-1} (base equivalent) respectively.

All stock solutions were prepared fresh on each day of the analysis and used within 30 min of preparation. Storage of solutions for longer periods is not recommended because of the relatively unstable nature of the drug. Prizidilol is also known to form complexes with a variety of metal ions [4] and pure nickel spatulas should be used for dispensing the drug.

All glassware was soaked overnight in a commercial detergent (5% w/v solution of Pyroneg in water), rinsed once in tap water and then twice in distilled water. Acetone should not be used for cleaning glassware as prizidilol reacts readily with aldehydes and ketones.

Collection and storage of biological samples

Human plasma for the preparation of standards was obtained by centrifugation (at 4°C) of blood from volunteer subjects and stored in 20 ml Sterivials at −20°C prior to use. Human urine was obtained and stored under similar conditions.

Blood from patients and volunteers who had received prizidilol was taken by syringe or cannula and transferred to tubes containing the anti-coagulant, lithium heparin. Each sample was gently mixed and then centrifuged to separate the plasma, which was transferred to plain tubes and quick-frozen over solid carbon dioxide. The samples were stored at −20°C prior to analysis. Urine samples were collected, and after volume measurement, sub-samples were quick-frozen in plain tubes and stored at −20°C until analysed.

Analysis of plasma — derivatization and extraction

To 1.0 ml of plasma in a polypropylene centrifuge tube (12 ml), was added 100 μl of the internal standard solution (equivalent to 2.00 μg SK&F 93238 base) and the samples were mixed by vortex. If less than 1 ml of sample was available, the volume was made up with control plasma, if possible from the same individual. The tubes were allowed to stand at room temperature for 5 min, then 2 ml of trichloroacetic acid (5% w/v aqueous solution pH 1) was added, the samples were mixed by vortex, and immediately centrifuged at 2000 ***g*** for 5 min. [It should be noted that the time between mixing and centrifugation was critical. If this period exceeded 5 min, preferential loss of the internal standard occurred.]

The supernatants were poured into polypropylene screw cap centrifuge tubes (12 ml) and 100 μl of the anisaldehyde solution was added. The samples were mixed by vortex and then incubated for 1 h in a shaking water-bath maintained at 70°C. After cooling to room temperature, the pH was adjusted to 12 by the addition of about 200 μl sodium hydroxide solution (4 mol l^{-1}). The pH was checked with narrow range pH papers (Merck, Darmstadt, B.R.D.; Alkalit Range pH 7.5–14).

Dichloromethane (4 ml) was added to each tube and the samples were extracted for 10 min on a blood cell suspension mixer. Following centrifugation for 5 min at 2000 ***g***, the aqueous layer was removed by aspiration and discarded. The organic layer was transferred to polypropylene tubes (12 ml) and evaporated to dryness in a vortex evaporator (Buchler Instruments, NJ, USA) which was maintained under reduced pressure (375 mmHg) for 1 h at 25°C. The evaporated solvent was collected in a cold trap containing a mixture of solid carbon dioxide and propan-2-ol.

The residues were reconstituted in 100 μl methanol–water–glacial acetic acid (75:25:0.1 v/v). At this stage the samples could be stored overnight at −20°C prior to analysis by HPLC.

A standard curve was produced by spiking aliquots of plasma (1 ml), obtained from individuals who had not received prizidilol, to cover the range 0.00–1.00 mg l^{-1} equivalent of SK&F 92657 base. Each standard sample was processed in the same manner and at the same time as the test samples.

Analysis of urine

To 1 ml of urine in a screw-cap polypropylene centrifuge tube (12 ml) was added 100 μl of the internal standard solution for urine (equivalent to 3.00 μg SK&F 93238 base) and the tubes were mixed by vortex. If less than 1 ml of sample was available the volume was made up with control urine, from the same individual if possible. Each tube remained at room temperature for 5 min, then 2 ml of trichloroacetic acid (5% w/v aqueous solution) was added and the samples were mixed by vortex. Anisaldehyde solution (100 μl) (1% in methanol) was added, and the samples were processed as described for plasma.

A standard curve (range 0.00–5.00 mg l^{-1} equivalent of SK&F 92657 base) was obtained by assaying spiked samples of urine (1 ml), obtained from individuals who had not received a dose of prizidilol. Each standard sample was processed in the same manner and at the same time as the test samples.

HPLC equipment

The chromatograph consisted of a Waters Associates Model 6000A pump coupled to a Waters W.I.S.P. auto-injector and a Perkin–Elmer LC-75 UV detector. The detector was operated at a wavelength of 326 nm and set to give 0.02 or 0.08 absorbance units full scale (a.u.f.s.) for the plasma and urine assays respectively.

The analytes were separated by a stainless steel column 150 mm × 4.6 mm i.d., packed with Zorbax-CN (6 μm particles, DuPont).

HPLC solvent

Methanol was filtered through a 0.45 μm membrane (Millipore, type FH) and distilled water through a 0.50 μm membrane (Millipore, type HA) prior to use. To make 500 ml of the HPLC solvent, 375 ml methanol and 125 ml distilled water were mixed in a round-bottom flask (1 litre) and degassed under reduced pressure. To this was added 0.16 ml *n*-dibutylamine and 0.16 ml orthophosphoric acid (88%). The column was allowed to equilibrate with the solvent for at least 1 h. After use the column was flushed with methanol–water (75:25 v/v) for a minimum of 1 h.

Chromatography

Using a mobile phase flow rate of 3 ml min^{-1}, the retention times for SK&F 92657 anisaldehyde hydrazone and SK&F 93238 anisaldehyde hydrazone were 3 and 4 min respectively. Typical chromatograms from plasma and urine extracts are shown in Figs 2 and 3.

Quantification

Peak area measurements were obtained using a Laboratory Data Control 301 computing integrator. The ratio of the drug peak area to internal standard peak area was

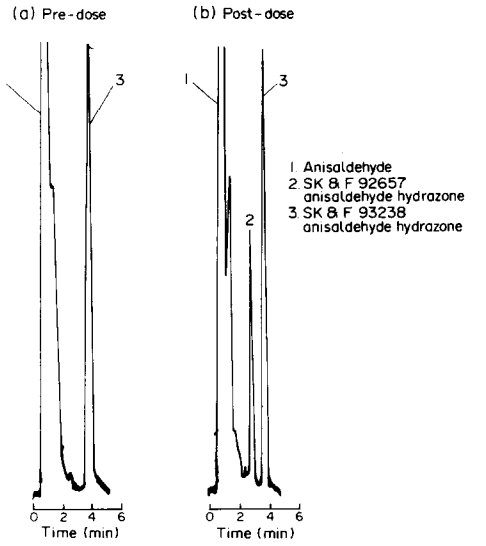

Figure 2
Typical chromatograms of extracted human plasma taken before and after administration of SK&F 92657-A_2.

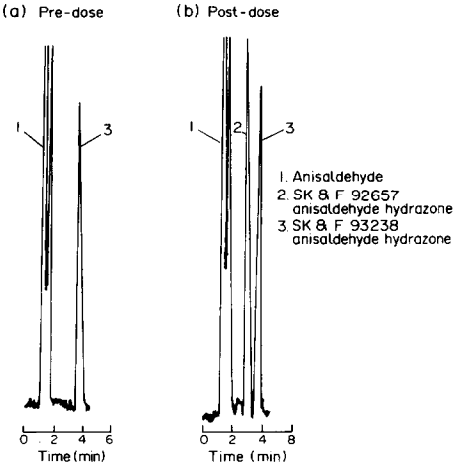

Figure 3
Typical chromatogram of extracted human urine taken before and after administration of SK&F 92657-A_2.

plotted against the known concentrations of prizidilol in the standards. A linear regression was obtained and the unknown concentrations read directly from the standard curve. The curve was found to be rectilinear over the concentration range 0–10 mg l^{-1} in plasma and 0–50 mg l^{-1} in urine assays. The slope, intercept and correlation coefficient for plasma and urine were 1.62, 0.02, 0.988 and 0.40, −0.03, 0.996 respectively.

Studies with ^{14}C-prizidilol

Samples of plasma and urine were spiked with ^{14}C-prizidilol and assayed as previously described. The final residues were reconstituted in methanol–water–acetic acid and were then taken up in 10 ml Picofluor 15 scintillant; the recovery of ^{14}C was determined by radiocounting in a Denley Mark III spectrometer.

Results and Discussion

Precision and accuracy

The precision and accuracy of the assay were assessed on three separate days by spiking ten replicate samples of plasma and urine each day with known concentrations of prizidilol.

The precision, expressed as the relative standard deviation (R.S.D.) was acceptable at 1 mg l^{-1} (10%) for plasma analysis, but the assay was less precise (18%) at 0.1 mg l^{-1}. The accuracy expressed as percentage error was 2% at 1 mg l^{-1} and 10% at 0.10 mg l^{-1}. For urine the precision was better (4% at 5.00 mg l^{-1} and 11% at 0.50 mg l^{-1}). The accuracy for this assay of prizidilol in urine was excellent, having percentage errors of 2% and 0% for concentrations of 5 and 0.5 mg l^{-1} respectively. The method is considered to be sufficiently accurate and precise for levels of prizidilol likely to be encountered in samples from patients and volunteers.

Recovery

For samples of plasma containing ^{14}C-prizidilol (0.10–1.00 mg l^{-1}) the mean recovery was 53%. For urine samples containing ^{14}C-prizidilol (0.50–5.00 mg l^{-1}) the mean recovery was 95%. The poorer recovery of drug from plasma can be explained by losses due to occlusion by, or binding to, precipitated plasma protein.

The amount of internal standard recovered from plasma and urine samples was determined from calibration curves constructed from the peak height response after injection of known amounts of SK&F 93238 anisaldehyde hydrazone onto the chromatograph.

The recovery of the internal standard was 27% from plasma and 59% from urine. The lower recovery of the internal standard from plasma can also be explained by losses due to occlusion by or binding to precipitated plasma proteins.

The recovery of both the drug and the internal standard from plasma has been shown to be directly proportional to the time taken between addition of the trichloroacetic acid and centrifugation. The rate of loss of the internal standard was greater than for the drug and therefore it is essential that this time period is reduced to a minimum and kept constant for all samples and standards. The recovery of the drug and internal standard from urine, however, was not dependent upon the time between addition of the acid and centrifugation.

Specificity

Many pre-dose plasma and urine samples have been assayed and no endogenous compounds have been found which interfered with the chromatography of either the anisaldehyde hydrazone of prizidilol or the corresponding hydrazone of the internal standard.

Although the metabolism of prizidilol in man has not been fully elucidated, one can postulate that the major metabolites might include acetylation products or hydrazones, following reaction with endogenous aldehydes and ketones. Similar reactions of the hydrazine moiety of hydralazine are well documented [5]. Under the conditions of the assay these metabolites would be converted back to parent drug. The assay is therefore not specific and will measure parent drug and these acid-labile metabolites.

Stability of prizidilol in biological fluids

Knowledge of the stability of prizidilol in biological fluids was required so that samples could be stored, whilst awaiting analysis, without affecting the results of the assay. The stability of the drug in plasma and urine was determined at room temperature and −20°C by spiking aliquots of plasma and urine with prizidilol and assaying replicate samples at pre-determined time intervals. The results for plasma indicated that the drug was stable for 6 h at room temperature and for one week at −20°C. Plasma samples should therefore be stored at −20°C if they are not to be assayed on the day of collection. However, even at −20°C plasma samples cannot be stored for longer than 7 days without changes in the drug concentrations. The results from stability studies of prizidilol in urine at room temperature and at −20°C showed that samples should be analysed within 2 h of collection or stored at −20°C. Although the drug was stable in urine at concentrations of 1.25 and 3.75 mg l^{-1} over a 5-week period at −20°C, it must be stressed that both the plasma and urine data reflect only the stability of the parent drug in the absence of metabolites and may not give a true assessment of the breakdown of prizidilol and metabolites, when these are both present in a sample.

Use of the assay in experimental studies

The assay has been used to determine the plasma and urine concentration of prizidilol and its acid-labile metabolites in studies of the compound in man and experimental animals. These studies include pharmacokinetic investigations following oral and intravenous administration of the drug to human volunteers, and the bioequivalence of different formulations. A plasma concentration–time curve following intravenous infusion of prizidilol in a human subject is shown in Fig. 4. Despite the lack of selectivity, this assay has aided the selection of dose formulations and regimens for clinical and toxicological trials.

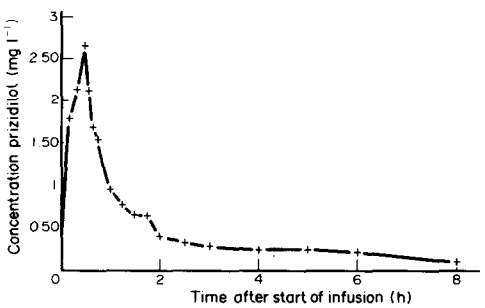

Figure 4
Plasma concentration–time curve for a single subject following a 30 min infusion of prizidilol (0.8 mg kg^{-1}).

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