Improved Performance of the Second Generation α₁-AGP Columns:
Applications to the Routine Assay of Plasma Levels of Alfuzosin Hydrochloride

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ABSTRACT
Described is a direct enantioselective separation of the enantiomers of alfuzosin hydrochloride on the second generation α₁-AGP column which offers improved efficiency, shorter analysis, and improved stability with respect to the first generation columns. The method has been applied to the analysis of drug substance in rat plasma. This highly efficient extraction method and the use of fluorimetric detection result in selective and sensitive determination of the enantiomers. The analytical validation parameters demonstrate the applicability of this method to pharmacokinetic and metabolic studies.

KEY WORDS: stereochemistry, enantiomers, direct separation, alfuzosin

INTRODUCTION

Among the commercially available chiral columns, α₁-acid glycoprotein (AGP) phases were found to be very useful for the direct enantioselective separations of a variety of compounds of pharmaceutical interest.¹⁻³ AGP is the main cationic binding protein in the human organism. It is a glycoprotein with a relative molecular mass of 41,000 with sialic acid incorporated in the sugar part. Its native form AGP has an isoelectric point of 2.7.

The separations on these phases are based on the stereodifferentiating properties of proteins which constitute the basis of affinity chromatography. The predominant interactions are electrostatic (ionic) and hydrophobic, although charge-transfer and hydrogen bonding may also be operative on these phases.

The first generation of AGP-silica-based columns was available as EnantioPac® cartridges (100 × 4.0 cm, 10 μm particles) from LKB-Pharmacia (Bromma, Sweden). According to the manufacturer, these phases were prepared by adsorption of AGP to the diethylamino-functionalized silica with large pore volume and surface area by ionic binding. The primary alcohol groups of the sugar residues were then oxidized to aldehyde groups by sodium metaperiodate. The latter groups were cross-linked with the amino groups of the protein. The resulting imino groups were then reduced with sodium cyanoborohydride to give hydrolytically stable bonds.

These columns suffered from several drawbacks. The maximal flow rate was limited to 0.3–0.5 ml/min, and the only organic modifier compatible with the column was 2-propanol, the proportion of which in the mobile phase could not exceed 10% (v/v). The recommended amount of solute injected was of the order of 1–5 nmol.

For maximum column life they had to be operated in the pH range of 3.5–7.0 and at temperatures below 45°C. The most commonly used buffer was phosphate (0.01–0.04 M) with cationic (amines, quaternary ammonium salts) or anionic (aliphatic organic acids) additives.³

In addition to the excessively long equilibration times due to low operational flow rates and restricted choice of organic modifiers, the first generation AGP columns suffered from slow mass transfer phenomena which often resulted in broad peaks and thus low detection sensitivity. The maximal column efficiencies were of the order of several hundred theoretical plates per column length. Furthermore, poor mechanical stabilities and short lifetimes precluded their use in low-term studies of biological samples.

The second generation AGP columns based on a patented process of immobilization of α₁-AGP on 5 μm spherical silica particles with a much smaller pore volume and a smaller surface area, which were commercialized approximately a year ago, have considerably better mechanical stability.⁵ The large differences in mechanical and chemical stability between the first and second generation columns are due to the properties of the silica matrix and its surface chemistry. The latter columns can be used at increased flow rates (up to 0.9 ml/min) and with organic modifiers such as 1-propanol, methanol, ethanol, and acetonitrile, in addition to 2-propanol at

Received for publication May 10, 1989; accepted May 22, 1989.
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concentrations up to 15% (v/v). Thus a much wider choice of mobile phase selectivities can be obtained.

We have previously reported on the use of the first generation AGP columns in the analysis of drug enantiomers. However, the current availability of the second generation AGP columns has enabled us to improve the separation of alfuzosin hydrochloride and to develop a method of sufficient ruggedness for pharmacokinetic studies.

Alfuzosin (N-(3-(4-amino-6,7-dimethoxy-2-quinazolinyl)methylamino)propyl)tetrahydro-2-furancarboxamide hydrochloride (1) is a highly selective antagonist of α1-adrenergic receptors used in the treatment of hypertension and benign hypertrophy of the prostate. Its pKₐ value is 8.1 and its partition coefficient (log P) is 1.5 at pH 7.4.

The drug product is marketed as a racemate. However, in the course of drug development it was important to dispose of an enantioselective separation method in order to study the pharmacokinetic and metabolic profiles of the drug.

$$\text{HCl}$$

$$\begin{align*}
\text{H}_3\text{C} & \quad \text{O} \\
\text{N} & \quad \text{N} \\
\text{N} & \quad \text{NH} \\
\text{O} & \quad \text{CH}_3 \\
\text{CH}_3 & \quad \text{O} \\
\text{O} & \quad \text{NH}_2 \\
\text{CH}_3 & \\
\end{align*}$$

$$\text{HCl}$$

**MATERIALS AND METHODS**

**Reagents and Chemicals**

The chemicals, all of which were used in this study without prior purification, were obtained from the following sources: anhydrous potassium dihydrogen phosphate (ref. 4873, Merck, Darmstadt, F.R.G.), tetrabutylammonium bromide (ref. 19.311-9 from Aldrich Chemical Co., Milwaukee, WI), 1 M sodium hydroxide, ready to use, 1 M hydrochloric acid, ready to use, and dichloromethane were from Prolabo (Paris, France), methanol RS ACS (ref. 414902) and ethyl ether RPF (nonstabilized, ref. 447534) were from Carlo Erba (Milan, Italy). Purified water was obtained with a Millipore Milli-Q purification system (Milford, MA).

**Apparatus and HPLC Conditions**

The HPLC equipment consisted of a Waters Assoc. automatic injector (Wisp, model 712B), a Jasco 880-PU pump, a Jasco spectrofluorimeter model 4200, and a Spectra-Physics calculator/integrator model 4200. The chromatographic column was a Chiral-AGP (100 × 4.0 mm i.d, 5 µm) from ChromTech (Huddinge, Sweden).

The mobile phase was a mixture of 0.05 M potassium dihydrogen phosphate containing 0.025 M tetrabutylammonium bromide adjusted to pH 7.4 with 1 M sodium hydroxide and acetonitrile (94:6, v/v). The flow rate was 0.9 ml/min. The optimal excitation and emission wavelengths were 265 and 400 nm, respectively.

**Sample Preparation**

A 1.0 ml aliquot of rat plasma sample was spiked with µl of a methanolic solution of the internal standard 2 prepared at a concentration of 2 mg/ml. The samples were vortex mixed, and 1 ml of 0.1 M NaOH was added. To each tube was added 7 ml of a mixture of dichloromethane and ethyl ether (nonstabilized) (3 + 4, v/v), and the samples were stirred for 25 min. Next, the samples were centrifuged at 1000g for 5 min at 4°C. The organic phase was withdrawn and transferred to another test tube, evaporated to dryness under a stream of nitrogen at 30°C. The residue was dissolved in 80 µl of the HPLC mobile phase and vortex mixed. A 20 µl sample was analyzed.

$$\begin{align*}
\text{H}_3\text{C} & \quad \text{O} \\
\text{O} & \quad \text{C} \\
\text{N} & \quad \text{N} \\
\text{N} & \quad \text{NH}_2 \\
\text{CH}_3 & \quad \text{N} \\
\text{O} & \quad \text{N} \\
\text{H}_2 & \quad \text{C} \\
\end{align*}$$

**RESULTS AND DISCUSSION**

Figure 1A illustrates the separation of a sample of porcine blank plasma and the same sample spiked with alfuzosin and the internal standard 2 which is derived from the same chemical family of compounds. Since the internal standard does not contain a chiral center, it gives a unique, well-defined peak which elutes close to but well resolved from the peaks of the enantiomers of alfuzosin. Baseline resolution of the two enantiomers and the internal standard was obtained and the peak asymmetry factors were approximately 1.7. The R-isomer was eluted before the S-isomer. The total analysis time was less than 10 min. The efficiency of the column was of the order of 1500 plates/column.

The use of fluorimetric detection combined with the extraction procedure ensures high selectivity of the method: no interferences of the endogenous compounds in the extracts were observed. Plasma extracts containing the enantiomers of alfuzosin dissolved in the HPLC mobile phase were stable for at least 48 h at room temperature and for several months at −20°C.

The response–concentration curve was studied by analyzing spiked plasma samples in the concentration range of interest. The detection limit for the two enantiomers is of the order of 1 ng/ml for the sample size of 1 ml of plasma; the coefficient of variation at this concentration is less than 10%. The precision of the assay was studied by two analysts on two days by analyzing plasma samples spiked with alfuzosin at concentrations corresponding to 5 and 50 ng/ml. The average within-assay and
In conclusion, we have developed an improved HPLC enantioselective method using a new-generation AGP column. This column offers the advantages of improved efficiency, shorter analysis, longer lifetime, and mechanical stability, and it can be used with a wider choice of organic modifiers. The analytical validation parameters and the robustness of the method demonstrate its applicability to multisample analysis needed in pharmacokinetic and metabolic studies.

ACKNOWLEDGMENTS

The authors are grateful to Dr. Alexander Wick, Director of the Chemistry Department of Synthelabo Recherche, for his support and the Pharmacokinetic group for the biological samples.

LITERATURE CITED