Development and Validation of an Reversed-Phase Liquid Chromatographic Method for Analysis of Spiramycin and Related Substances

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Summary

A simple, robust, sensitive isocratic liquid chromatographic (LC) method suitable for the analysis of spiramycin is described. This method utilizes XTerra RP18 as the stationary phase and acetonitrile-0.2M K2HPO4 (pH 6.5)-water, 39.5:5:55.5 (v/v), as mobile phase. The mobile phase flow rate is 1.0 mL min⁻¹; the column is maintained at 70 °C by immersion in a water-bath; and UV detection is performed at 232 nm. The method has good selectivity towards the major components (spiramycins I, II, and III), other related substances, and many impurities. The robustness of the method was evaluated by means of full-factorial experimental design.

Introduction

Spiramycin, a macrolide antibiotic derived from the fermentation broth of Streptomyces ambifaciens, is a 16-membered lactone ring to which mycaminose and forosamine sugars are attached at positions 5 and 9, respectively, via β-glycosidic bonds [1]. The major components, spiramycins I, II, and III, contain mycarose sugar attached at position 4 of the mycaminose moiety. Some of the minor related components, for example neospiramycins I, II, and III, do not contain mycarose. Figure 1 shows the chemical structures of spiramycin and structurally related substances, and the abbreviations used throughout this text.

Several reversed-phase LC methods have been described for the analysis of spiramycin [2–10]. Some [2–7] have focused on the separation of the major components (SPM I, SPM II, and SPM III) only. The method described by Horie et al. could be used to separate these components and degradation products such as NSPMs I, II, and III, although separation of the main component (SPM I) was incomplete [8]. The work of Liu et al led to the separation of six spiramycins, the NSPMs, and several unknown related substances, thus confirming the complex composition of spiramycin [9]. Although selectivity was good, the efficiency and sensitivity of this method were rather poor, because of the poor efficiency of the poly(styrene-divinylbenzene) stationary phase. The official method prescribed by the European Pharmacopeia (Ph. Eur.) utilizes reversed-phase C8 silica gel with a mobile phase of pH 2.2 containing sodium perchlorate [10]. Although these conditions result in better efficiency, resolution of the major peak from the surrounding impurities is incomplete and the method is reported to be affected by the quality of the acetonitrile used in the mobile phase and in the solvent used to dissolve the samples. This might be responsible for the appearance of a ghost peak with a retention time of 1.1 relative to that of SPM I [11].

A capillary electrophoresis (CE) method utilizing phosphate buffer with a mixture of sodium cholate and CTAB was found to be less selective, and only suitable for the separation of the major components of spiramycin and four of the related compounds [12].

It was, therefore, decided to develop a method that would combine the advantages of the LC methods mentioned above, i.e. the selectivity of the polymer method with the efficiency of the official method. This laboratory has recently obtained very good selectivity and sensitivity by using XTerra C18 as stationary phase for the analysis of other macrolides, e.g. erythromycin and troleandomycin [13–15]. In contrast with classical silica-based packings, this stationary phase is based on silica gel containing methyl groups – to reduce the number of silanol groups on the silica gel surface. The organosilane substituent contains a polar carbamate group, and it is claimed that this stationary phase is more chemically and thermally stable.
This paper describes a simple, selective, sensitive, robust, and linear isocratic reversed-phase LC method utilizing XTerra C18 which enables the separation of SPM I from related substances and from several other minor components, at levels down to 0.05% m/m.

Experimental

Reagents and Samples

Acetonitrile, HPLC-grade S, was from Biosolve (Valkenswaard, The Netherlands). Dipotassium hydrogen phosphate (Merek, Darmstadt, Germany) 0.2 M solution was adjusted to the required pH by addition of 0.2 M phosphoric acid (Merek). Methanol, HPLC grade, was from HyperSolv (Poole, UK). pH 2.2 buffer solution was prepared from phosphoric acid and sodium hydroxide (Merek), according to the Ph. Eur. [10]. Sodium perchlorate was from Merek. Water was distilled twice from glass apparatus.

SPMs I, II, III, and IV and NSPM I and NSPMs II and III (mixture) were available as in-house standards [9]. Spiramycin chemical reference substance of the European Pharmacopoeia (Ph. Eur. CRS, 4489 L.U. mg⁻¹) was obtained from the European Pharmacopoeia Laboratory (Strasbourg, France).

LC Instrumentation and Chromatographic Conditions

LC was performed with a Merck-Hitachi (Darmstadt, Germany) L-6200 Intelligent pump, a Gilson (Villiers-le-Bel, France) type 234 autosampler equipped with a 20-µL loop, a Spectra-Physics (Fremont, CA, USA) Linear UVIS variable wavelength detector, which was operated at 232 nm, and a Hewlett-Packard (Avondale, PA, USA) model HP 3396 series II integrator. Compounds were separated on a Shandon (Runcorn, UK) 250 mm × 4.6 mm i.d. Hypersil BDS C8 5μm column, maintained at 25 °C, (Ph. Eur. Method) and on a Waters (Milford, MA, USA) 250 mm × 4.6 mm i.d. RP18 5μm XTerra column, maintained at 70 °C; temperature was controlled by means of a Julabo (Seelsbach, Germany) thermostat immersed in a water-bath. Acetonitrile-0.2 M K₂HPO₄ pH 6.5-water, 39.5:5:55.5 (v/v) at a flow rate of 1.0 mL min⁻¹ was used as the mobile phase. The mixture was degassed by purging with helium. The injection volume was 20 µL.

Results and Discussion

Method Development

Previous results, obtained for troleandomycin and erythromycin, indicated that a neutral mobile phase pH favoured the separation of these macrolides on XTerra RP18 [13–15]. Mobile phases containing different amounts of acetonitrile (x%, v/v), as organic modifier, 0.2 M phosphate buffer, pH 7.0 (5%, v/v), and water (95–x%, v/v) were, therefore, used as the starting conditions for method development on XTerra RP18. Under these conditions SPM II and SPM III were separated from SPM I but SPM I coeluted with neospiramycin III and an unknown minor impurity; unknown minor components eluted adjacent to SPM II and SPM III. Several other minor components eluted close to the column dead volume as a cluster; these were followed by elution of NSPM I, NSPM II, and SPM IV – polar components which were not completely separated. To improve the overall selectivity optimum organic modifier, pH, and column temperature conditions were studied.

The effect of pH on the separation was investigated between 5.5 and 8.5 at 60 °C with a mobile phase containing 40% (v/v) acetonitrile. At pH 8.5 separation was excellent but elution was too slow – the main component (SPM I) was eluted after 50 min. Better selectivity and efficiency, as characterized by the separation of several minor, unknown components, was obtained. A faster system was not investigated further, because the continuous use of pH 8.5 at high temperature was found to be unsuitable [14] – under these conditions column lifetime was too short (peak fronting was normally observed as the first symptom and this was followed by peak-broadening). A change of pH from 8.5 to 5.5 resulted in reduction of the separation time and rapid elution. This was as expected, because at lower pH spiramycins (pKₘ 7.6–7.7) are more protonated and hence less retained by a reversed-phase system. Between pH 5.5 and 6.0 SPM II was incompletely separated from the other minor components and therefore a mobile phase pH 6.5 was chosen for method development.

Sample Preparation

Spiramycin Ph. Eur. CRS at a concentration of 1.0 mg mL⁻¹ was used throughout method development. Different solutions containing 0.1 mg mL⁻¹ SPM I, SPM II, SPM III, SPM IV, NSPM I, and a mixture of NSPMs II and III were prepared for identification purposes. An artificial mixture containing all these components was also prepared for the robustness study. Acetonitrile-water, 3:7 (v/v) was used as solvent.