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# Automated method for the determination of a new matrix metalloproteinase inhibitor in ovine plasma and serum by coupling of restricted access material for on-line sample clean-up to liquid chromatography

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## Abstract

A fully automated liquid chromatographic method was developed for the determination of Ro 28-2653, a new synthetic inhibitor of matrix metalloproteinases (MMPs), in ovine serum and plasma. The method was based on the coupling of a pre-column packed with restricted access material, namely LiChrospher RP-8 ADS (alkyl diol silica), for sample clean-up to an analytical column containing octyl silica stationary phase. One hundred μl of biological sample, to which 2-propanol was automatically added, were injected onto the ADS pre-column, which was then washed with a washing liquid consisting of a mixture of 25 mM phosphate buffer (pH 7.0) and acetonitrile (90:10; v/v) for 10 min. By rotation of the switching valve, the analyte was then eluted in the back-flush mode with the LC mobile phase composed of a mixture of acetonitrile and 25 mM phosphate buffer (pH 7.0) (57:43; v/v). The UV detection was performed at 395 nm. The main parameters likely to influence the sample preparation technique were investigated. The method was then validated over a concentration range from 17.5 to 1950 ng/ml, the first concentration level corresponding to the lower limit of quantitation. At this concentration level, the mean bias and the R.S.D. value for intermediate precision were −2.4% and 4.2%, respectively.

*Keywords:* Matrix metalloproteinase inhibitor; Ro 28-2653; Plasma; Serum; Sample preparation; Column-switching; Restricted access material; Liquid chromatography

## 1. Introduction

Ro 28-2653 (Fig. 1) is a new synthetic inhibitor of matrix metalloproteinases (MMPs) [1–5]. The ability of these enzymes to degrade various components of the extracellular matrix seems to play a major role in tumor invasion and metastasis [1]. Among the inhibitors of MMPs recently synthetized, Ro 28-2653 presents a high selectivity towards MMP2, MMP9 and membrane type 1-MMP [2,4]. This promising compound is able to reduce tumor growth in orthotopic prostatic cancer in rats. A significantly prolonged survival of the treated rats was also demonstrated [2]. In addition, other antitumor and antiangiogenic effects have been reported [4].

Ro 28-2653 is very poorly soluble in water. This low solubility estimated at about 0.56 μg/ml in water at 25 °C gives rise to difficulties in the pharmaceutical formulation of oral or injectable solutions [5]. Nevertheless, aqueous solutions of Ro 28-2653 have been formulated due to the good solubility of the complex obtained with a β-cyclodextrin derivative [5].

As far as we know, only one method has been described for the liquid chromatographic (LC) determination of Ro 28-2653 in rat plasma [2]. Prior to LC analysis, the sample preparation technique was liquid–liquid extraction. When the

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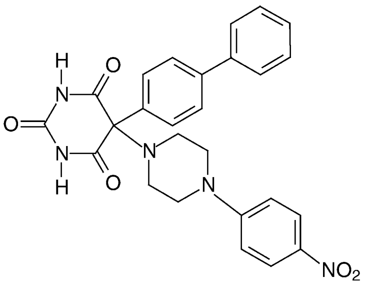

Fig. 1. Structure of Ro 28-2653.

number of biological samples to be analysed is particularly large, such as the bioavailability and pharmacokinetic studies, this manual procedure becomes tedious and time-consuming.

Therefore, in order to determine the bioavailability of Ro 28-2653 in sheep after oral and intravenous administration of a solution and to compare the main pharmacokinetic data obtained with those observed after oral administration of a suspension, a fully automated method was developed for the LC determination of this compound in plasma and serum. Sample clean-up was performed by on-line coupling of a pre-column packed with restricted access material (RAM) to the analytical column by means of the column-switching technique. Among the restricted access sorbents, a family belonging to the group of internal surface reversed-phase (ISRP) supports, namely alkyl diol silica (ADS), packed in pre-columns has been applied successfully for the clean-up of plasma samples prior to LC analysis [6–14]. The topochemistry of the ADS sorbents has been represented schematically elsewhere [6,8,13,15,16]. Due to the appropriate pore diameter (about 6 nm) of the silica particles, macromolecules, such as proteins, having a molecular mass larger than 15 kDa, are excluded in the void volume of the pre-column and are directly flushed into the waste. Moreover, some hydrophilic and electroneutral diol groups bound to the external surface of the particles prevent the adsorption and denaturation of proteins. On the other hand, low molecular mass compounds, such as the analytes of interest, have a free access to the internal surface of the support, on which butyryl ($C_4$), capryloyl ($C_8$) and stearoyl ($C_{18}$) moieties are bonded, and can be retained mainly by hydrophobic interactions.

The aim of this paper was to develop a fully automated method using this kind of RAM for on-line sample clean-up prior to the LC determination of Ro 28-2653 in ovine plasma and serum. The main parameters likely to influence this sample preparation technique were studied. The determination of the retention capability of the ADS support, the optimisation of the composition of the washing liquid, the selection of the most suitable times for the rotation of the switching valve were considered. The influence of the addition of a protein releasing reagent to the biological sample on the chromatographic peak shape was also investigated. The method was then validated according to a novel approach based on accuracy profiles taking into account the total measurement error [17,18].

## 2. Experimental

### 2.1. Chemical and reagents

Ro 28-2653 was kindly supplied by Roche Diagnostics (Penzberg, Germany) and was used without further purification. Phosphoric acid (85%; m/m), hydrochloric acid (37%; v/v), sodium hydroxide, 2-propranol were purchased from Merck (Darmstadt, Germany) and were of analytical grade. Random methyled β-cyclodextrin (Wacker, Burghausen, Germany) was also used during method development. Methanol and acetonitrile were LiChrosolv LC gradient grade solvents obtained from Merck. The water used in all experiments was purified by means of a Milli-Q system (Millipore Corporation, Bedford, MA, USA).

The pre-column containing LiChrospher RP-8 ADS (particle size: 25 μm) was supplied by Merck. The analytical and guard columns were pre-packed with LiChrospher 60 RP-Select B (particle size: 5 μm) from Merck.

### 2.2. Apparatus

An ASTED XL module from Gilson (Villiers-le-Bel, France), designed for the on-line coupling of dialysis and trace enrichment to LC, has been modified into a column switching system. It consisted of an auto-sampler equipped with one model 401C dilutor fitted with a 1 ml syringe and two Rheodyne model 7010 six-port switching valves (Berkeley, CA, USA), one of which being connected to a Manu-CART system containing the LiChroCART pre-column (25 mm × 4 mm, i.d.) packed with LiChrospher ADS material from Merck. The other switching valve was equipped with a 100 μl injection loop. A replaceable in-line filter (2 μm, sieve) contained in a holder from Alltech (Deerfield, IL, USA) was also installed between the injection loop and the pre-column.

The LC system was composed of a model 305 pump coupled with a model 805 manometric module (Gilson) and of a model Dynamax UV-1 variable-wavelength UV–vis absorbance detector (Rainin, Woburn, MA, USA). A model 306 pump from Gilson was also used for sample preparation.

The separation was performed on a LiChroCART column (125 mm × 4 mm, i.d.) preceded by a LiChroCART guard column (4 mm × 4 mm, i.d.), both contained in a Manu-CART system from Merck. They were thermostated at 35 ± 0.1 °C in a model 12B/VC Julabo waterbath (Seelbach, Germany).

The "715 HPLC system Controller" software loaded on an IBM compatible computer (CPU type Pentium) and the "722 Keypad" software were used for the control of the LC and the modified ASTED XL systems, respectively. The e.noval® software (Arlenda, Belgium) was used to obtain the accuracy profiles as well as all validation results.

### 2.3. Chromatographic conditions

The chromatographic separation was performed in the isocratic mode. The mobile phase consisted of a mixture of