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Development of an *in vitro* liquid chromatography–mass spectrometry method to evaluate stereo and chemical stability of new drug candidates employing immobilized artificial membrane column

Giuseppe Cannazza[a,*,1], Umberto M. Battisti[a,1], Marina M. Carrozzo[b], Addolorata S. Cazzato[a], Daniela Braghiroli[a], Carlo Parenti[a], Luigino Troisi[b]

[a] Department of Life Sciences, University of Modena & Reggio Emilia, Via Campi 183, 41125 Modena, Italy
[b] Department of Biological and Environmental Sciences and Technologies, University of Salento, via Prov.le Lecce-Monteroni, 73100 Lecce, Italy

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ABSTRACT

A stopped-flow HPLC method was developed to evaluate configurational and chemical stability of pharmaceutical compounds employing immobilized artificial membranes (IAM) column to simulate conditions that pharmaceutical compounds will meet *in vivo*. The method was applied to recent developed chiral 5-arylbenzothiadiazine derivatives possessing high positive allosteric modulatory (PAM) activity on AMPA receptor. In particular the stopped-flow HPLC method developed used a chiral column to separate single enantiomer of the compounds that are forced into an IAM column where configurational and chemical stability was evaluated in simulated gastrointestinal fluids (pH 1.2 and 6.8 at 37.5 °C) to simulate *in vivo* conditions. The results were compared to those obtained by dynamic and off-column methods to evaluate the effects of stationary phases on kinetic constant of enantiomerization and hydrolysis. The results suggested that the phospholipids environment of IAM stationary phases, which mimes biological membrane, greatly influence the hydrolysis process increasing the chemical stability of tested compounds while no influence on enantiomerization kinetic was observed. Therefore it is possible to suppose that 5-arylbenzothiadiazine derivatives should not hydrolysed *in vivo* while they should rapidly racemized in aqueous solvents. The method could represents a rapid and value tool to predict chemical and configurational stability of new chemical entities to decrease the number of animal studies.

1. Introduction

Chemical and stereo stability are one of the most important properties of drug candidates and it has a marked influence on drug discovery and development. Chemical degradation of a pharmacological active compound can lower the intended concentration below the therapeutic dose. On the other hand, decomposition can generate toxic molecules and thus possible side effects. Poor bioavailability of an active pharmaceutical ingredient can be also related to instability. Compounds may also not be active when dosed orally, owing to instability in the pHs or enzymes of the gastrointestinal system. Some of the most common degradation pathways involve racemization and hydrolysis.

For this reason, U.S. Food and Drug Administration (FDA) requires extensive stereo chemical investigation on chiral drugs; consequently, it is important to understand the stereochemical integrity of those compounds that are administered as pure enantiomers [1,2]. Indeed, stereoinversion of chiral labile compounds could be studied by different techniques such as dynamic NMR (DNMR), chirooptical methods, off-column and on-column chromatographic methods [*i.e.*, dynamic gas chromatography (DCG), dynamic high-performance liquid chromatography (DHPLC), dynamic capillary electrophoresis (DCE), and stopped-flow HPLC (sfHPLC)] [3–19]. Among dynamic chromatographic methods the DCX-Explorer software, developed by Trapp et al., is one of the widest technique employed and it takes advantage of the unified chromatography equation [17–27].

Similarly hydrolysis processes has been observed for several drugs and it is generally evaluated dissolving the compounds in buffers miming gastrointestinal biofluids that are analysed by chromatographic methods like thin-layer chromatography (TLC), high-performance thin-layer chromatography (HPTLC), gas

* Corresponding author. Tel.: +39 059 2055136; fax: +39 059 2055750.
E-mail address: giuseppe.cannazza@unimore.it (G. Cannazza).
[1] The authors contributed equally to the work.

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chromatography (GC) and HPLC [28]. Hydrolysis can occur for compounds containing specific functional groups such as: ester, amide, thioester, imide, imine, carbamate, acetal, alkyl chloride, nitrate, lactam, lactone, and sulphonamide [29–31]. Raection kinetic can be influenced by pH of dissolution solvents or by the presence of enzymes. Examples of hydrolysis include aspirin, which contains an ester and hydrolyzes to salicylic acid and acetic acid and chloramphenicol, which contains an amide group that hydrolyzes to the corresponding amine and dichloroacetic acid [29–31].

Due to the importance of stability studies, they are usually conducted in the early drug discovery process. Since stability in solution state is the main focus in drug discovery, these studies typically include stability in gastrointestinal fluids and bioassay media. Anyway stability test in solutions don't resemble *in vivo* conditions due to absence of biological membranes that could influence stability kinetics.

Herein we reported a new method to evaluate simultaneously stereo and chemical stability of a selected class of compounds, in gastrointestinal fluids and in conditions that mime those found *in vivo*. This new technique combines stopped-flow bidimensional recycling chromatographic system (sf-BD-rHPLC), an immobilized artificial membrane (IAM) column and mass spectrometry.

Sf-BD-rHPLC permits to perform stereo and chemical stability studies in solvents similar to physiological fluids and not in mobile phase that contains organic modifier that could influence the obtained results [32–34]. Moreover sf-BD-rHPLC method allows to choose the appropriate stationary phase in which stability studies will be conducted. Since pharmaceutical compounds are in contact with biological membranes and dissolved in physiological solutions when administered *in vivo*, stability can be evaluated under the same conditions in order to obtain reliable stability data. With this aim stationary phases that simulate biological membranes could be employed to evaluate the chemical and configurational stability under experimental conditions that resemble those *in vivo*. In IAM stationary phase composed of lecithin monolayers (phosphatidylcholine), wherein each lipid molecule is covalently linked to propylamine/silica, became commercially available, enabling this kind of experiments [35–44].

Recently our research group has designed and synthesized a series of 5-arylbenzothiadiazine derivatives (Fig. 1) with an interesting activity as positive modulator on AMPA receptor (AMPA-PAM) [45]. Since computational and experimental studies suggested that only one enantiomer of this type compounds possess AMPA-PAM activity, it becomes relevant to resolve the racemic mixtures in order to identify the eutomer. Anyway previous studies on chiral benzothiadiazine type compounds showed stereo and chemical liability in aqueous acidic solvent suggesting to evaluate stereo and chemical stability of the new prepared AMPA-PAM compounds before considering the activity of the single enantiomers *in vivo* [32–34,46–53]. Thus the new developed technique has been applied to evaluate the stability of the compounds **1–4**. The results were also compared to those obtained by dynamic and off-column methods to evaluate the effects of stationary phases on kinetic constant of enantiomerization and hydrolysis.

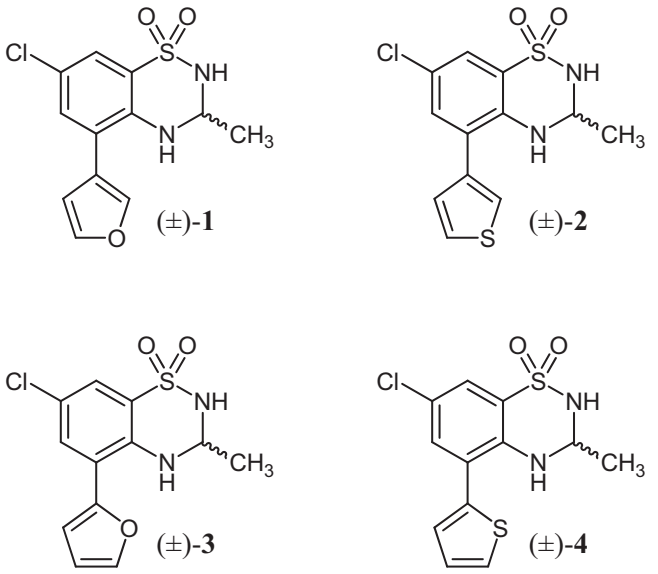

Fig. 1. 5-Arylbenzothiadiazine derivatives. 7-Chloro-5-(3-furanyl)-3-methyl-3,4-dihydro-2*H*-1,2,4-benzothiadiazine 1,1-dioxide (**1**), 7-chloro-5-(3-thiophenyl)-3-methyl-3,4-dihydro-2*H*-1,2,4-benzothiadiazine 1,1-dioxide (**2**), 7-chloro-5-(2-furanyl)-3-methyl-3,4-dihydro-2*H*-1,2,4-benzothiadiazine 1,1-dioxide (**3**) and 7-chloro-5-(2-thiophenyl)-3-methyl-3,4-dihydro-2*H*-1,2,4-benzothiadiazine 1,1-dioxide (**4**).

2. Experimental

2.1. Material and reagents

HPLC analysis were carried out on a chromatographic apparatus composed of a Shimadzu LC-10AD Pump (Shimadzu Italia, Milan), a Merck Hitachi L-6200A Pump (Merck KGaA, Darmstadt, Germany), a Rheodyne 7725 manual injector equipped with a 20 μl sample loop (Jasco Europe, Italy, Milan). A Merck Hitachi L-7400UV (Merck KGaA, Darmstadt, Germany) was used as detector. Chromatograms were recorded with a Jasco J-700 program (Jasco Europe, Italy, Milan). Two Rheodyne 7000 valves were used to switch the mobile phase flow (Jasco Europe, Italy, Milan). Column temperature regulation was obtained with a Jasco CO-2067 column oven (Jasco Europe, Italy, Milan).

The columns used were Chiralcel OD-RH [cellulose tris (3,5-dimethylphenylcarbamate); 250 × 4.6 mm I.D.; 5 μm], Chiralcel OD [cellulose tris (3,5-dimethylphenylcarbamate); 250 × 10 mm I.D., 10 μm] purchased from Chiral Technologies Europe, Illkirch, France, SUPELCOSIL C8 (150 × 4.6 mm I.D., 5 μm), purchased from Supelco, CHIRASPHER NT (250 × 10 mm I.D., 5 μm) purchased from Merck, IAM Column (Immobilized artificial membrane) (150 × 4.6 mm I.D., 10 μm).

Melting points were determined with an Electrothermal Apparatus and they are uncorrected. ^{1}H NMR spectra were recorded with a Brucker DPX 400 spectrometer with $CDCl_3$ as solvent and tetramethylsilane (TMS) as external standard. Chemical shifts (δ) are in part per million and coupling constant (J) in hertz. Multiplicities are abbreviated as follows: s, singlet; d, doublet; dd, double doublet; t, triplet; m, multiplet.

LC–MSMS experiments were carried out on an Agilent 1200 series LC and interfaced to an Agilent 6410 triple-quadrupole mass spectrometer equipped with an electrospray ionization source. All data were acquired and analysed using Agilent MassHunter Quantitative Analyses version B.01.04 analyst data processing software.

All pH measurements were made using Orion Research EA940 pH-meter. HPLC-grade acetonitrile, *n*-hexane and 2-propanol were obtained from Sigma-Aldrich (Milan, Italy).

2.2. Chemistry

2.2.1. 2-Amino-5-chloro-3-(3-furanyl)benzensulfonamide

The compound was obtained as described by Battisti et al. [45]. Yield 70% (two steps), m.p.: 81–83 °C.^{1}H NMR (400 MHz, $CDCl_3$): δ = 5.10 (s, broad, 2*H*), 5.12 (s, broad, 2*H*), 6.56 (s, 1*H*), 7.32 (d, J = 2.5 Hz, 1*H*), 7.57 (t, J = 1.5 Hz, 1.6 Hz, 1*H*), 7.63 (s, 1*H*), 7.75 (d, J = 2.5 Hz, 1*H*). GC–MS (70 eV):*m*/*z* 272 (84)[M+], 191 (67), 163 (60), 128 (100), 101 (30).