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Research paper

Screening therapeutics according to their uptake across the blood-brain barrier: A high throughput method based on immobilized artificial membrane liquid chromatography-diode-array-detection coupled to electrospray-time-of-flight mass spectrometry

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ABSTRACT

The Blood-Brain Barrier (BBB) plays an essential role in protecting the brain tissues against possible injurious substances. In the present work, 79 neutral, basic, acidic and amphoteric structurally unrelated analytes were considered and their chromatographic retention coefficients on immobilized artificial membrane (IAM) stationary phase were determined employing a mass spectrometry (MS)-compatible buffer based on ammonium acetate. Their BBB passage predictive strength was evaluated and the statistical models based on IAM indexes and in silico physico-chemical descriptors showed solid statistics (r^2 $(n - 1) = 0.78$). The predictive strength of the indexes achieved by the MS-compatible method was comparable to that achieved by employing the more "biomimetic" Dulbecco's phosphate buffered saline, even if some differences in the elution order were observed. The method was transferred to the MS, employing a diode-array-detection coupled to an electrospray ionization source and a time-of-flight analyzer. This setup allowed the simultaneous analysis of up to eight analytes, yielding a remarkable acceleration of the analysis time.

1. Introduction

Nowadays synthetic combinatorial [1], parallel libraries [2] or purified solutes extracted from natural products allow medicinal chemists to obtain hundreds of new chemical entities (NCEs) at an incredibly fast rate. This large amount of NCEs is subsequently profiled according to their pharmacokinetic, pharmacodynamic (PK/PD) [3] and their toxicological properties. Indeed, this process drives and impacts the whole drug discovery path, being financially hazardous and often jeopardized by remarkably high rates of failure [4]. Moreover, the drug discovery for neurotherapeutics, *i.e.* drugs targeting the central nervous system, suffers from a much higher attrition. In fact, only 3 to 5% of CNS drug candidates entering phase I clinical trials were successfully launched in the last years, compared to approximately 10% for all the other compounds [5]. This occurrence is due to the presence of the blood–brain barrier (BBB), which severely limits the entry of xenobiotics into the brain [6]. The discovery of the BBB dates back more than a century when, in 1885, Paul Ehrlich observed that trypan blue dye, when intravenously administered, stained all organs except for the brain and the spinal cord [7]. In 1913, Edwin Goldmann, an Ehrlich associate, demonstrated that when the same pigment was directly injected into the cerebrospinal fluid (CSF), it stained the nervous tissues but no other viscera [8]. Those findings clearly outlined the existence of a complex histological organization, segregating the brain parenchyma from the interstitial fluids [9]. Many years of research broadened the knowledge of the BBB and shed new light into the mechanisms that it adopts to protect the CNS integrity, which are indeed both physical and metabolic [10]. In fact, cerebral endothelial cells are connected by intercellular proteins such as occludins, claudins and junctional adhesion molecules, *i.e.* transmembrane proteins that are responsible for the

Abbreviations: AA, ammonium acetate; APC, Amemiya predictive criterion; BBB, blood-brain barrier; CNS, central nervous system; CSF, cerebrospinal fluids; DAD, diode array detector; DPBS, Dulbecco's phosphate buffered saline; ESI, electrospray ionization source; IAM, immobilized artificial membrane; LC, liquid chromatography; LOO, leave-one-out; MD, molecular dynamics; MLC, micellar liquid chromatography; MS, mass spectrometry; MW, molecular weight; NCE, new chemical entity; ODS, octadecylsilyl; PBS, phosphate buffered saline; PC, phosphatidylcholine; PD, pharmacodynamics; PK, pharmacokinetics; PLS, partial least squares; PSA, polar surface area; QSPR, quantitative structure-property relationship; TOF, time-of-flight; UV, ultraviolet; VIF, variance inflation factor

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formation of tight junctions [11], sealing the paracellular pathway and making the brain nearly inaccessible to polar compounds, unless they are the substrates of specific transporters [12]. The BBB also acts as a metabolic barrier due to the presence of numerous enzymes. The outcome of their activity can be (*i*) the conversion of potentially harmful drugs into inactive CNS compounds, (*ii*) the activation of harmless drugs into their active CNS metabolites or (*iii*) the degradation of the therapeutics into metabolites or substrates of specific efflux transporters, such as the P-glycoprotein or multidrug resistance proteins [13].

Therefore, drugs can cross the BBB mainly by passive transcellular diffusion [14] and less frequently by active uptake [15]. While the former can be modeled starting from physicochemical properties of the compound, such as lipophilicity, molecular weight (MW), molecular volume and ionization state, the latter is described by their affinity to specific transporters (influx and efflux). These exist permanently within the membrane and span the lipid bilayers across which they transport substances. There are two types of active transport: primary and secondary active transport. While the primary active transport requires the hydrolysis of ATP, the secondary active transport uses an electrochemical gradient. However, the involvement of active transporters is hardly predictable on a merely structural basis and a drug design oriented to deliberately target them is not advantageous. This is because the uptake of xenobiotics *via* active transporters can be heavily altered by other ongoing medications and is saturable, thus resulting in an inconstant, inadequate, and unpredictable hematic drug concentration *vs* time profile [16].

The PK of drugs is greatly influenced by their membrane barrier passage, *i.e.* the rate and the extent at which they cross biological membranes [17]. This passive diffusion mediated drug passage has been for long assumed as dependent on the analyte *n*-octanol/water lipophilicity, expressed as log P, classically determined *via* the *shake-flask* method [18]. Apart from being tedious and time-consuming, this method accurately reflects, to a certain extent, the membrane partitioning of neutral compounds, but greatly underestimates that of the analytes supporting one or more electrical charges [19]. The octadecylsilyl (ODS)-based liquid chromatography (LC) estimation of drug passage provides a faster approach, but suffers from the same drawback, being that the interactions of the analytes with the stationary phase are mainly driven by the analyte molecular hydrophobicity [20]. This discrepancy subsists because neither *n*-octanol nor ODS support electrical charges as membrane phospholipids do. For sure cell-based assays reproduce more closely the characteristics of fluid membrane bilayers, offering a more realistic model [21]. However, the cellular assays, albeit predictive, are lengthy and require cell culturing skills. In addition, cell cultures have a certain degree of leakiness which in general exceeds that of the *in vivo* BBB [22]. This aspect might make the comparison among data achieved in different laboratories or employing slightly different methodologies misleading.

Immobilized artificial membrane (IAM)-LC combines the advantages arising from a more realistic biomimetic system to the increased reproducibility and robustness of LC [23–26]. IAM stationary phases are typically based on analogues of phosphatidylcholine (PC), the main constituent of eukaryotic cell membranes [27]. The use of IAM-LC for gaining indexes able to assist in the prediction and mechanism elucidation of drug passage through biological barriers, such as the BBB, has been extensively explored since the middle of nineties [28–30]. Recently, a work by our research group [31] proposed a medium throughput method aimed at developing statistical models for an improved prediction of the BBB uptake of drugs and the derived models showed remarkable statistics. However, each analyte had to be determined separately due to the low selectivity of the ultraviolet (UV) detection, thus requiring significant working time. Thereby the need of handling accurate screening methods faster such as to process the samples in a more effective and reliable way emerged.

The aims of the present study are therefore:

1. The development of an IAM-LC method with higher throughput as compared to those already reported in the literature [31,32];
2. The study of the influence of the employed buffers both on the retention and on the BBB passage predictive strength of the derived indexes;
3. Its validation in terms of BBB crossing potential by developing statistical models for a prediction of the extent at which drugs cross the BBB;

To achieve these goals the following arrangements were undertaken:

1. The chromatographic conditions were carefully calibrated to become mass spectrometry (MS)-compatible and fast. At first, the determinations were carried out employing UV detection and the BBB predictive strength of the models was evaluated. Subsequently, the LC was coupled to a diode-array-detection (DAD) and to an electrospray ionization (ESI)-time-of-flight (TOF) – MS to make the most of the higher selectivity given by *m/z ratio* and the compounds were analyzed simultaneously in a mixture;
2. The throughputs of the present method and of two others developed by the same research group [31,32] were compared to evaluate if, and to which extent, an improvement was achieved;
3. Steady-state blood to brain distribution ratio values (log BB), which are the logarithms of the ratios between the concentrations that the drugs realize in the brain tissue and those they realize in the blood, were used as references to validate the method and demonstrate its suitability;

Indeed, log BB is the most common parameter used to quantify penetration of a compound across the BBB and, since it is measured at equilibrium, is an index of BBB permeability [33]. Log BB values are experimentally derived *in vivo*, usually in rodents. For these determinations, a drug is administered to a rat and, after the reach of equilibrium, its concentration is measured in the brain and in the blood. The prevalence of this parameter can be attributed to the fact that it is well understandable for medicinal chemists and easier to measure compared to kinetic BBB permeability data (*e.g.* log PS [33]). Several authors noted that a drug may be considered CNS permeable if its log BB value exceeds a certain threshold (usually 0) [34], albeit caution is needed as low values of this parameter may sometimes suggest an extensive plasma protein binding. The phosphate buffered saline (PBS) eluents, routinely employed in the IAM-LC determinations as able to mirror more closely the physiological microenvironment, are not compatible with MS detection. In fact, they may rapidly contaminate the ionization source, thus seriously suppressing sample signals [35]. Therefore, MS-compatible ammonium acetate (AA) based eluents were employed for the determination of IAM indexes of 79 structurally unrelated acidic, basic, neutral and amphoteric compounds. In addition, molecular dynamics (MD) experiments were carried out in water boxes offering the opportunity of studying the dynamic evolution of the system as well and deriving the properties of the most populated conformational states of the analytes of interest [36]. Furthermore, the influence played by the different buffers on the chromatographic retention and on the BBB passage predictive strength of the indexes was scrutinized. This study was performed to find out whether using AA based buffers rather than the conventional PBS affected in any noticeable extent the predictive strength of the models, and if so, if this was a reasonable price to pay to the higher selectivity and the increased throughput of the technique.

2. Materials and methods

2.1. Chromatographic columns

All the experiments were performed on a Regis IAM Fast Mini Screening (10 μm, 10 mm × 3.0 mm; Morton Grove, IL, USA) analytical