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Bioanalysis of small-molecule drugs in nasal and paranasal tissues and secretions: Current status and perspectives

Review Article

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Abstract: Over the last years, interest in intranasal administration as an alternative and promising route for the delivery of drugs with local, systemic, and even central nervous system action has tremendously increased. Accordingly, understanding of the properties and characteristics of the nasal cavity as well as the biodisposition processes of drugs into the nasal compartments is acquiring a significant prominence in the field of pharmacology. In this context, the development and validation of bioanalytical methodologies for the quantitative measurement of drugs and their metabolites in nasal and paranasal tissues and/or secretions is of the utmost importance. However, currently, information concerning bioanalysis of drugs in nasal and paranasal tissues and/or secretions is scattered. This review aims to provide a valuable overview of the methodologies that have been used for the collection and preparation of nasal and paranasal samples with special emphasis placed on the review of liquid chromatographic methods employed for the quantitative determination of small-molecule drugs and their metabolites in such specimens.

Keywords: *Nasal and paranasal tissues and secretions • Bioanalysis • Liquid chromatographic methods*

1. Introduction

Over the last few years, the nasal pathway has raised an increasing interest within the scientific community for to its potential to be a promising route for the administration of drugs as an alternative to oral and parenteral routes [1-5]. Conventionally, intranasal drug delivery has been used for the symptomatic relief or treatment of local disorders such as nasal congestion, allergy or infection [6-8]. However, the large surface area associated with the extensive vascularisation of the nasal mucosa affords optimal conditions for the rapid and extended absorption of therapeutic compounds in the nasal region, therefore allowing systemic delivery of drugs administered through this route [1,2,9]. In fact, rapid absorption, avoidance of gastrointestinal destruction and hepatic first-pass metabolism, quick pharmacological onset of action and high systemic availability are the positive attributes that contribute towards the feasibility of the intranasal drug administration as a valuable option to overcome the limitations and handicaps related to oral and parenteral routes [5,10-13]. Moreover, the intranasal drug administration is non-invasive, painless and easy to perform which maximizes patient convenience, comfort and compliance [7]. From a pharmacokinetic viewpoint, it is widely recognized that the distribution of systemically administered drugs to the central nervous system (CNS) usually remains a great challenge, mainly due to the anatomical and physiological protective nature of the blood-brain barrier (BBB) [14]. Therefore, recent developments in the field of neurosciences have encouraged the discovery of new therapeutic strategies to achieve a more effective pharmacological treatment of prevalent neurological diseases such as sleep disorders, epilepsy, Alzheimer's and Parkinson's. In particular, assuming the olfactory region as a unique

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direct connection between the nose and the brain, the intranasal administration has emerged as a promising approach for the delivery of therapeutic agents to the CNS bypassing the BBB [13-19].

A wide variety of therapeutic agents including both small molecules and macromolecules, like peptides and proteins, can be successfully delivered by this route. Some examples of nasal drug formulations are already available in the market. Among these are, for example, budesonide (Rhinocort®), mometasone (Nasonex®), butorphanol (Stadol NS®), nafarelin (Synarel®), desmopressin (Desmospray®), oxytocin (Syntocinon®) and salmon calcitonin (Miacalcin®) [3,6,7,20].Undoubtedly, the increasing exploration of the intranasal route for topical, systemic, or direct CNS drug delivery offers attractive perspectives in the near future. Taking into account the continuous development in the pharmaceutical field and the great amount of ongoing studies assessing the potential of well-known drugs for intranasal administration, it is expected that in the next few years a range of novel nasal drug formulations will reach the market. For instance, dimenhydrinate [21], sildenafil [22], erythropoietin [23], levodopa [24], metoclopramide [25], zidovudine [26], sumatriptan [27], ropinirole [28], nitrendipine [29], insulin [30], ondansetron [31], zolmitriptan [32], olanzapine [33] and carvedilol [34] are examples of conventional drugs belonging to different pharmacotherapeutic groups that have been recently considered for intranasal drug administration. As it occurs in usual drug discovery and development (DDD) programs, bioanalysis is also a decisive scientific branch to support the development of new formulations incorporating conventional drugs intended for intranasal administration. Obviously, blood and urine are the conventionally collected biological samples for bioanalytical purposes in DDD programs. However, to thoroughly investigate the drug efficacy and safety, the analysis of drugs and/or metabolites in other biological specimens are often required. Therefore, in certain circumstances, the collection of tissues or secretions is considered an appropriate approach to better understand the pharmacokinetic properties of new drug candidates or of well-known drugs incorporated in new pharmaceutical formulations. Nevertheless, additional challenges such as the limited availability of tissue samples or fluids and the invasive nature of the collection procedures are commonly found. Hence, in these cases, the bioanalytical considerations that need to be taken into account are different from those required for conventional samples such as blood (whole blood, serum or plasma) and urine [35,36].

Thus, in the absence of a publication that systematizes the different procedures dealing with bioanalysis in nasal/paranasal tissues and secretions, this review will offer a concise description of the procedures and methodologies currently employed for the collection and preparation of nasal samples. It aims to highlight the liquid chromatographic techniques used for separation, detection and quantification of small-molecule drugs in these specimens.

2. Bioanalysis in nasal and paranasal region

Bioanalysis is the term generally used to describe the set of analytical techniques and procedures applied in the characterization and quantification of bio-compounds, drugs and metabolites in biological matrices such as blood, serum, plasma, urine or other fluids and tissues [37,38]. It plays a significant role in the evaluation and interpretation of bioavailability, bioequivalence, pharmacokinetic and toxicokinetic studies [39]. The importance of bioanalysis in nasal and paranasal samples has enhanced tremendously in parallel with the progressively higher therapeutic impact attributed to nasal route for the administration of topical, systemic, and CNS-acting drugs. In general, the assessment of the drug disposition in plasma/serum and in tissues like liver and kidneys is essential to a better understanding of the pharmacokinetic profiles (drug concentration *versus* time post-dose). However, the determination of drugs and/or metabolites at the site of action (biophase) is also important.

The increasing prevalence of upper respiratory tract infections (URTI) like acute and chronic rhinosinusitis (inflammation of nasal mucosa and paranasal sinuses) has motivated the development of new formulations incorporating antihistaminic, corticosteroid and antimicrobial agents for intranasal delivery [40]. Indeed, the intranasal administration of such medicines will enable the direct drug delivery in biophase, aiming to improve the clinical efficacy and to minimize the systemic toxicity associated with such therapeutic agents administered *per os*. In this context, it is essential to understand the pharmacokinetic behaviour of drugs (*e.g.* metabolism and mucocilliary clearance) after intranasal drug delivery, not only in nasal tissues and/or secretions (as a predictor of drug efficacy), but also in blood, serum or plasma samples in order to evaluate the extent of systemic drug exposure (as a predictor of drug safety).

Currently, pharmacokinetic and pharmacodynamic studies are integral and often decisive components of drug development and evaluation. Inclusively, pharmacokinetic/pharmacodynamic (PK/

PD) modelling provides valuable data on the time course of pharmacological effect of a given dose, allowing the definition of appropriate dosing regimens [41]. For practical reasons, PK/PD assessments have often been confined to the measurement of drug concentrations in matrices which are easy to obtain like blood, serum, plasma or urine. However, the total plasma concentrations is not an ideal pharmacokinetic parameter for rational dosing of drugs, since it may under- or over- estimate the drug concentration in the target site, providing a less realistic prediction of its clinical efficacy [41-43]. Unfortunately, technical difficulties in collecting sufficient amounts of nasal and paranasal samples remain the major handicap in pharmacokinetic studies. For instance, in order to overcome nasal sampling limitations, Bimazubute and co-workers [44] investigated the possibility of predicting the concentrations of enrofloxacin in pig nasal secretions, knowing its concentrations in plasma. This study demonstrated that enrofloxacin concentrations in nasal secretions could be satisfactorily predicted from the plasmatic levels by establishing an equation in which the two variables were correlated. Although these results are promising, no other evidence has been demonstrated so far. Thus, further investigation is needed on this matter.

Bearing in mind that URTI's biophase is the nasal compartment, the determination of drug/metabolite concentrations in nasal and paranasal areas is of great interest to evaluate local drug exposure and tissue penetration and, consequently, the therapeutic success [44-54]. Effectively, the measurement of drug concentrations in nasal secretions [45,55,56] and structural tissues such as nasal mucosa [46,57], paranasal sinus mucosa [47-49], ethmoid bone [46] and septal cartilage [58] has been performed not only in intranasal drug delivery but also after oral, intravenous or intramuscular drug administration. Several studies have been published suggesting that the analysis of nasal and/or paranasal samples can provide useful information from a pharmacokinetic viewpoint, nonetheless, valuable contributions have also been found in the fields of toxicology [59-63] and pathophysiology [64-71].

Given the relevance that the quantitative determination of drugs in nasal and paranasal samples has for the management of inflammatory and infectious conditions of the upper respiratory tract, the development of proper analytical methodologies to support reliable pharmacokinetic assays is crucial. Since the quantity of nasal samples collected is often limited, the total amount of drugs/metabolites in such specimens is expected to be low, therefore, special features regarding sample collection, sample preparation, chromatographic analysis, and validation procedures should be considered. In the following sections, the main issues related to the various stages of the bioanalytical methods employed for the quantitative analysis of drugs/metabolites in nasal and paranasal specimens will be discussed.

2.1. Sampling of nasal and paranasal tissues and secretions

Practical sampling difficulties are a major problem when trying to assess drug penetration into nasal and paranasal cavities. The limited availability of nasal tissues and secretions leads to the need for optimization of the sample collection and processing methodologies in order to ensure that sufficient amounts of truly representative samples are available to perform reliable quantitative analyses. This section will provide a comprehensive overview of the various techniques and procedures frequently used to collect samples from nasal and paranasal regions. In order to afford a better understanding of the specimens involved, the anatomical characteristics of the nasal cavity will be also briefly addressed.

2.1.1. Nasal anatomy

The nose is a structurally and functionally complex organ whose primary functions are breathing and olfaction in both humans and animals. The human nose is divided longitudinally by a cartilaginous nasal septum in two symmetrical and non-connected halves; each one opens at the face through nostrils extending posteriorly to the nasopharynx. Anatomically, the human nasal cavity consists of three main regions, which are the nasal vestibule, the respiratory region and the olfactory region (Fig. 1, I.). The nasal vestibule is the most anterior part of the nasal cavity and corresponds to the region just inside the nostrils with an area of about 0.6 cm^2 [6]. The respiratory region is the largest area of the nasal cavity (≈150 cm^2) [7,72] comprising three nasal turbinates (also called conchae): the inferior, the middle and the superior, that are projected from the lateral nasal walls making part of a convoluted and folded structure [6,73]. Surrounding the nasal cavity there are paired air-filled spaces, the paranasal sinuses, which act as auxiliary chambers to heat and humidify the inspired air. According to their location, paranasal sinuses are divided in maxillary sinus, frontal sinus, sphenoid sinus and ethmoid sinus [74] (Fig. 1, I. and II.).

2.1.2. Nasal tissues

The collection of nasal and paranasal tissues is an invasive procedure and, for this reason, involves the use of topical or general anaesthesia. In humans, harvesting

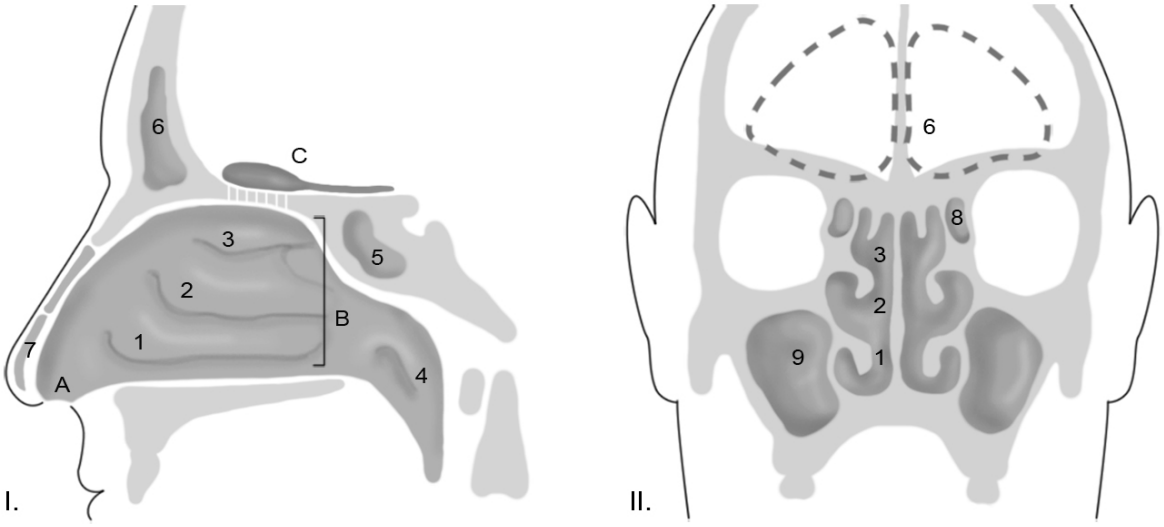

Figure 1. Schematic representation of the human nasal cavity. I. Longitudinal cross-section; II. Transversal cross-section; A. Nasal vestibule; B. Respiratory region; C. Olfactory region; 1. Inferior turbinate; 2. Middle turbinate; 3. Superior turbinate; 4. Nasopharynx; 5. Sphenoid sinus; 6. Frontal sinus; 7. Septal cartilage; 8. Ethmoid sinus; 9. Maxillary sinus.

of the nasal tissue is usually accomplished by biopsy [75] or by upper respiratory tract surgical procedures (*e.g.* endoscopic sinus surgery) [46,48,53,57], whereas in animals the removal of tissue specimens can only be attained after their sacrifice [76-78]. Once removed, mucosal tissues are carefully separated from bone and cartilage debris. Generally, before the weighing and processing, all the specimens are gently blotted with absorbent paper or rinsed with isotonic saline solution to remove surface blood [46,47,75,79].

Since tissue samples are obtained at the time of surgery, each subject is only available for one tissue sample collection [50]. Moreover, local mucosal effects, such as bleeding, are commonly associated with biopsies which make repeated tissue sample collection procedures from the same nasal cavity problematic [75,80]. Hence, the assessment of drug penetration into nasal/paranasal tissues has traditionally been based on single point estimates that are used to calculate the ratio of drug concentrations at the site of action relatively to serum/plasma. In addition to sampling procedures of nasal secretions not being adequate to specify the distribution of drugs throughout the various nasal and paranasal compartments [81], there is evidence suggesting that nasal secretion fluid may not accurately represent the effective drug concentrations in tissues [82]. However, it is broadly accepted that collecting nasal secretions constitutes a valuable tool to support drug penetration studies in nasal/paranasal regions. It also allows for repeated sampling over time from the same individual and, thereby, helps with consistent characterization of the pharmacokinetic profiles [83,84].

2.1.3. Nasal secretions

Nasal secretions are a heterogeneous fluid mainly composed of a mixture of cells, plasma exudation and mucus [85] and are characterized by significant variations between individuals with respect to the amount, composition, cellular content and physical properties [86]. Unlike other biological fluids such as plasma or urine, the collection of nasal secretions requires special attention and coordinated efforts since the method selected for sampling directly influences the experimental results obtained [86]. Indeed, different collection techniques yield heterogeneous matrices and distinct analyte concentrations, which make the data comparison between studies difficult [86]. To date, several techniques for the collection of nasal secretions have been described; however, none of them have been generally accepted as ideal. Accordingly, a great effort has been spent to standardize and validate the collection techniques of nasal secretions in order to achieve more uniform, reliable and reproducible results. Standard methodologies to collect nasal secretions have been proposed by some authors [80,85,87-89].

Currently, techniques used to sample nasal secretions can be typically categorised into four different groups according to their principles [90]. Thereby, non-invasive methods to harvest nasal secretions can be divided for collection of spontaneous secretions, aspiration, absorption and washing techniques. Taking into account that only a very thin layer of secretion exists on the nasal mucosa, the collection of sufficient amount of sample to perform the analysis is commonly difficult, especially when healthy subjects are involved [90]. Therefore, all

Table 1. Advantages and limitations of sampling methods used to collect nasal secretions [51,85-87,90,92].

Sampling method	Characteristics	Advantages	Limitations
Collection of spontaneous secretions **- Nose blowing**	Collection of spontaneous nasal secretions based on blowing out the nose	• Simple • Specialized devices are not needed	• Variable sample amounts • Low sample volume (healthy subjects) • Restricted to hypersecretion conditions
Aspiration techniques **- Suction and microsuction** **- Indwelling catheter**	Aspiration of the secretions by the insertion of a tube into the nasal cavity	• Undiluted samples • Serial collection of nasal secretions (indwelling catheter)	• Low sample volume • Long time sampling • Inconvenient for the patient • Nasal mucosa irritation/damage • Sample contamination with blood
Absorption techniques **- Cotton strips** **- Filter paper strips or disks** **- Nasal swabs** **- Cellular materials (rubber foam, polyurethane foam, surgical cellulose sponges)** **- Sinus packs**	Introduction of an absorptive material into the nasal cavity Centrifugation to extract the absorbed secretion fluid	• Simple • Sufficient amounts of undiluted nasal secretions • Easy handling • Minor subject cooperation	• Difficult use in cases of nasal polyposis, anterior septal deviation and narrow vestibulum nasi • Long time sampling • Nasal mucosa irritation/damage • Once removed from the nose, rapid sealing and centrifugation is required
Nasal washing techniques **- Nasal lavage** **- Nasal spray washing**	Instillation of a washing solution into the nose	• Sufficient sample volume • Little discomfort for the subjects	• Unknown/unpredictable dilution of nasal secretions • Subject cooperation

sampling methods represent a compromise between the need to obtain sufficient sample amounts and the desire to minimize the mucosa disturbance [90,91]. Each of them has its own advantages and limitations (Table 1), as is discussed below.

2.1.3.1. Collecting of spontaneous secretions

Nose blowing is the simplest method to collect spontaneous nasal secretions. Although it is conducive to subjects with hypersecretion conditions, the quantity of sample obtained is extremely variable and the use of this technique in healthy subjects is commonly impractical due to the insufficient amounts of spontaneously secreted fluid for analysis [86].

2.1.3.2. Aspiration techniques

Aspiration techniques include, amongst others, suction and microsuction methodologies. In general, the collection of nasal secretions is achieved by the insertion of a tube (silicone catheter for suction and capillary glass tube for microsuction) into the nasal cavity to aspirate the secretions adherent to the mucosa [90]. Both techniques usually provide small sample volumes and the number of times for sampling often needs to be increased. The main disadvantage of microsuction regards to the direct contact between rigid tubes and nasal mucosa, which may easily cause tissue damage and consequently, the contamination of sample with blood [90]. A recent approach to aspirate sinus fluid specimens using the indwelling sinus catheter was successfully employed in a few pharmacokinetic studies for the evaluation of the time course of drug effects in patients with acute maxillary sinusitis [51,92-94]. Thus, in the procedure, after the application of topical anaesthesia, a polyethylene catheter was inserted into the maxillary sinus using a puncture device. Despite being an invasive procedure, the serial acquisition of sinus secretions provides a better characterization of the concentration-time profile of drugs in the maxillary sinus enabling the construction of more precise relationships between the drug exposure and clinical response [51,92]. Moreover, as referred by Ambrose *et al*. [92], it seems that the total number of patients required to demonstrate therapeutic efficacy could be reduced. Although this group of subjects might be smaller, the requirement of continuous insertion of the indwelling catheter in the maxillary sinus for long periods of time (over 72 hours) appears to be the major disadvantage, once it may hamper the enrolling of patients and even compromise the accomplishment and feasibility of the studies [51]. The dilution of the collected sinus aspirates is also a limitation of the method; however, it can be easily overcome by the determination of the dilution factor, which will be further discussed.

2.1.3.3. Absorption techniques

The absorption techniques involve the introduction of a collector with absorptive properties into the nasal cavity to remove the secretions on the nasal epithelial layer. Several absorptive collectors such as cotton pledgets [56,95,96], filter paper disks [97], nasal swabs [44,45,98], cellular materials (like rubber foam [90], polyurethane foam [86,87] or surgical cellulose sponges) and sinus packs [85] can be used to collect specimens of nasal secretions. After about 10-20 min of remaining within the nose, the absorbent material is withdrawn and immediately centrifuged for the extraction of the secretions, otherwise the collector could dry out leading

to the loss of fluid and thus affecting sample recoveries [90]. Effectively, suitable performance of an absorptive collector mostly depends on the high recovery of the secretion fluid and on the inability for analyte retention [99]. While in the study carried out by Jaehde *et al*. [56] the recovery of enoxacin from cotton pads was between 84 and 99%, only approximately 41% of ciprofloxacin was recovered from the nasal cotton pledgets used by another research team [95]. In this latter study, some pledgets exhibited a decrease in weight after being removed from the nostrils (*i.e*., were partially retained) and were excluded from the analysis [95]. Thereby, the choice of an appropriate absorptive material as well as the optimization of the sample handling and processing methodologies has paramount importance during the development of sampling procedures for nasal secretions. In general, absorption techniques are simple, easy to handle and well accepted by the subjects. In addition to providing sufficient amounts of undiluted nasal secretions, the absorption techniques often require minor cooperation of the subjects, which is particularly useful for the collection of secretions in children, elderly or comatose patients [87,89]. On the other hand, some physiological disorders such as nasal polyposis, anterior septal deviation or narrow vestibulum nasi may hamper the insertion of the collectors into the nose and interfere with their absorption efficacy [85]. In these cases, nasal washing techniques have emerged as the most convenient alternative. Although several studies have shown that absorption methods are sensitive, reliable and reproducible tools for the collection of nasal secretions [44,85], it has been also described that the introduction of more rigid absorptive materials like sponges, foams or swabs can induce local irritation or damage of the nasal mucosa. Indeed, the anatomic configuration of nasal and paranasal regions, either in humans or animals, is characterised by very narrow and snaky cavities that are typically hard to approach. Upon this fact, the collection of nasal secretions must be carefully performed to avoid the lesion of nasal tissues and, therefore, the contamination of samples with blood which inevitably results in the alteration of the analytes concentration under investigation [80].

2.1.3.4. Washing techniques

Nasal lavage is a commonly used method to collect nasal secretions in clinical practice, since it is non-invasive, easy to perform, atraumatic and provides little discomfort for the subjects. Traditionally, nasal washing techniques consists of irrigation with 3-5 mL of a pre-warmed, sterile and isotonic saline solution (0.9% NaCl) into the nostrils and subsequent expelling of the nasal lavage fluid to an appropriate container few seconds later [81,100,101]. Surprisingly, although it is a simple method, inconsistent results across distinct experimental studies reveal that washing procedures need to be properly defined [88]. In fact, the volume and composition of the washing solution, the liquid temperature, the duration of contact between the solution and mucosa, the pressure of irrigating liquid and the position of the subject during sampling are some of the methodological parameters that can interfere with the results obtained [88]. In contrast to most of the collection methods aforementioned, nasal lavage technique often provides sufficient amounts of sample to perform analytical measurements. Nevertheless, the instillation of the nasal cavities with washing solution volumes indubitably results in the dilution of the nasal secretions samples. As a consequence, the concentration of the analytes frequently falls below the lower limits of detection in the assays, in comparison with other sampling methods like, for instance, the absorption techniques [87]. Furthermore, unknown fractions of the instilled fluid may be accidentally swallowed, lost from the nasal opening or absorbed through nasal mucosa leading to a significant variability of the quantities of sample collected and subsequently to unpredictable degrees of nasal secretions dilution, thus compromising the reproducibility of the technique [80,86]. For this reason, maximum cooperation from the subjects is required which is not always technically possible [89]. In order to minimize the dilution extent of nasal secretion fluid, some alternative nasal washing approaches have been proposed. Hence, nasal spray washing technique enables the delivery of small portions of solution from a spray-pumped dispenser, keeping the final volume of the collected lavage fluid considerably smaller than using conventional nasal lavages [90]. Likewise, in the sampling technique employed by Hayden *et al*. [52], nasal mucus specimens were collected through the instillation of only 3 to 5 drops of washing solution to obtain significantly concentrated samples of secretions. However, the unknown dilution of the collected nasal secretions samples still persists and poses a serious problem in terms of interpreting the measured concentrations of various substances in the specimens [102]. Clearly, accurate quantification of analytes in nasal secretion fluid can only be achieved by the adjustment of the concentrations with the correspondent factor of dilution. Thus, several strategies have been suggested using either endogenous or exogenous compounds as markers of dilution. Endogenous levels of albumin [88], secretory immunoglobulin A (sIgA) and total protein [89] have been commonly used to correct nasal secretions dilution by establishing an index between the concentrations of the target compounds and the marker [88,90]. However, only relative amounts instead of

absolute concentrations of the analytes of interest can be determined, restricting its application to comparative studies of the substance levels among subjects [103]. On the other hand, inflammatory conditions of the respiratory system may alter the vascular permeability of mucosa which may affect the concentrations of endogenous proteins in nasal secretions and, therefore, the reliability of the adjustment [103,104]. Urea has also been proposed as an endogenous marker of dilution, since by virtue of its small molecular weight and relatively non-polar nature, it rapidly diffuses across membranes suggesting that its concentration in nasal secretions is equal to plasma [105,106]. Unfortunately, published data reveal that urea levels could vary with the volume of the washing solution used as well as the duration of the dwelling time [107]. Moreover, the simultaneous collection of blood samples is always required. Theoretically, the use of exogenous substances as markers of dilution is a more promising approach. Inulin [108], lithium chloride [109] and radiolabeled albumin are some examples of exogenous compounds that have been used for this purpose. These substances, which are normally not found in nasal secretions, are added in known concentrations to the irrigation solution and their recovery is analysed in the collected fluid to calculate the dilution factor of the secretions. It should be pointed out that, despite its potential, it is hypothesised that these compounds may be partially absorbed across the mucosa during highly inflammatory conditions [103]. Therefore, further studies to validate the use of exogenous markers of dilution are needed.

2.2. Preparation of nasal and paranasal samples

Biological samples such as blood, serum, plasma, urine, tissues and secretions are highly complex matrices usually not directly compatible with high-pressure liquid chromatographic (HPLC) analysis. Therefore, sample preparation is one of the most important stages of the bioanalytical method development, since it includes both the isolation and pre-concentration of target compounds from the matrix, making them more suitable for separation, identification, detection and quantification in chromatographic systems [110].

As shown in Table 2, the extraction of drugs/metabolites from nasal and paranasal samples has been achieved either by protein precipitation (PP), liquid-liquid extraction (LLE) or solid-phase extraction (SPE). Regardless of sample type (tissues or secretions), the analysis of specimens from nasal/paranasal region usually requires some pre-treatment steps. Nasal secretions are a complex mixture of several components including cells, plasma exudation and mucus with slightly acidic pH values ranging from 5.5 to 6.5 [121]. The nasal mucus is a viscoelastic fluid composed of 95% water, 2% mucin (glycoprotein), 1% inorganic salts, 1% lipids and 1% of other proteins such as albumin, immunoglobulins, lysozyme and lactoferrin [20]. The viscous properties of nasal secretions are mostly due to the presence of mucine therefore, small variations of its content may lead to significant viscosity alterations [122]. Moreover, the sampling procedure used to collect nasal secretions can also affect the consistency of the specimens. Thus, if aspiration or absorption techniques are used, it is expected that the secretions obtained present a relatively high degree of viscosity which may hamper its pippeting. In fact, in most cases it may be necessary to consider a previous dilution of the samples [111] or even pulverization in liquid nitrogen [113] before further processing. The heterogeneous distribution of drugs in nasal secretions may induce misleading results, thereby the inclusion of homogenization procedures is often required in order to attain representative samples and ensure the reliability of the outcome.

Rigid tissue samples such as ethmoid bone and septal cartilage can be previously processed by freezing in liquid nitrogen and pulverization in a mortar with pestle [46,58]. In case of soft tissue samples (*e.g.* maxillary sinus mucosa) homogenization methodologies are obviously necessary and thus can be accounted as an essential preceding step of the sample extraction process. In general, a small mucosal tissue sample is excised and carefully weighed, only after being gently blotted with absorbent paper or rinsed with isotonic saline solution to remove surface blood. The tissues are commonly homogenized by means of appropriate instruments (*e.g.* Ultraturrax) and the addition of buffer solutions (*e.g.* phosphate and Krebs-Ringer buffers) is often accomplished to facilitate the homogenization and to optimize tissue and drug stability. In order to achieve a convenient recovery of the compounds of interest, the homogenization step is also frequently integrated in the sample extraction procedure following the inclusion of protein precipitating agents [46,48,79] or water-immiscible organic solutions [49]. For instance, in the study developed by Gehanno *et al.* [48] for the determination of moxifloxacin concentrations in human sinus mucosa, tissue samples were extracted by homogenization with a mixture of acetonitrile/phosphoric acid to precipitate matrix proteins; recovery values ranging between 94 and 99% were attained. The collection of nasal tissue samples has been extensively performed in order to assess the tissue penetration and bioavailability of a variety of anti-infectious and other pharmacological agents used in the treatment of upper respiratory tract disorders. Despite the indisputable

Table 2. Liquid chromatographic methods for quantitative determination of drugs in nasal and paranasal samples.

Drug	Sample (volume/weight)	Subject	Sample preparation	IS	Apparatus	Stationary phase	Mobile phase	Detection	Validation parameters	Ref.
Acyclovir	Nasal fluid (50 µL)	Horse	**LLE** [dichloromethane-isopropyl alcohol, 50:50 (v/v)]	Ganciclovir	LC	Polymer PLRP-S 100 (150 mm x 2.1 mm, 5 µm)	Gradient elution Phase A: 5 mM ammonium formate in HPLC H_2O Phase B: MeOH	MS/MS	**Calibration range:** 2-100 ng mL^{-1} **LOQ:** 2 ng mL^{-1} **LOD:** 0.05 ng mL^{-1}	[111]
Acyclovir	Nasopharyngeal mucus (50 µL)	Horse Pony	**LLE** [dichloromethane-isopropyl alcohol (50:50, v/v)]	Ganciclovir	LC	Nucleodur C_{18} (125 mm x 2 mm, 3 µm)	Gradient elution Phase A: 5 mM ammonium formate in HPLC H_2O Phase B: MeOH	MS/MS	**LOQ:** 2 ng mL^{-1}	[98]
Amoxicillin Clavulanic acid	Sinonasal mucosa	Human	NR	-	HPLC	ND	NR	NR	**LOQ** $_{(Amoxicilin)}$: 170 ng g^{-1} **LOQ** $_{(Clavulanic\ acid)}$: 80 ng g^{-1}	[54]
Azithromycin	Sinus mucosa (20 mg)	Human	**PP** [ACN]	Azaerythromycin	LC	Mac-Mod XDB-C_8 (50 mm x 4.6 mm, 3.5 µm)	0.02 M acetic acid/MeOH with 2% tetrahydrofuran (90:10, v/v)	MS	**Calibration range:** 1-100 ng mg^{-1} **LOQ:** 1 ng mg^{-1}	[50]
Azithromycin	Sinus fluid (50 µL)	Human	**LLE**	[D-3] Azithromycin	HPLC	NR	NR	MS/MS	**Calibration range:** 10-500 ng mg^{-1}	[51]
Azithromycin	Sinus fluid Sinus mucosa	Human	NR	-	HPLC	NR	NR	Electron capture	**Calibration range:** 50-5,000 ng mL^{-1}	[82]
Bepotastine besilate	Nasal mucosa (24 mg)	Human	NR	-	LC	Capcell Pak MG II C_{18} (150 mm x 2.0 mm, 3 µm)	NR	MS/MS	**LOQ:** 2 ng g^{-1}	[57]
Budesonide	Nasal mucosa (3-25 mg)	Human	Microwave extraction	-	HPLC	NR	NR	MS/MS	NR	[75]
Cefetamet	Maxillary sinus mucosa (100-300 mg)	Human	**PP** [perchloric acid]	-	HPLC	Spherisorb ODS (125 mm x 4 mm, 1.5 µm)	4 mM perchloric acid/ACN (83:17, v/v)	UV (265 nm)	**Calibration range:** 300-50,000 ng mL^{-1} **LOQ:** 300 ng mL^{-1}	[79,112]
Cefotiam	Sinus secretions (200 µL)	Human	**PP and LLE** [ACN and dichloromethane]	-	HPLC	LiChrospher RP-18 (125 mm x 4 mm, 5 µm)	1000 ml H_2O + 80 ml ACN + 2 ml acetic acid, pH 5.1 adjusted with 10 M sodium hydroxide	UV (254 nm)	**Calibration range:** 50-2,000 ng mL^{-1} **LOQ:** 50 ng mL^{-1}	[113,114]
Cefpodoxime	Sinus mucosa (20 mg)	Human	**SPE** [Bond Elut C_8]	Cefaclor	HPLC	Supelcosil C_{18} (250 mm x 4.6 mm, 5µm)	0.05 M acetate buffer, pH 3.8/MeOH/ACN (86:12:2, v/v/v)	UV (235 nm)	**Calibration range:** 250-2,000 ng g^{-1} **LOQ:** 130 ng g^{-1} **Recovery:** ≈ 55%	[115]
Ceftibuten	Nasal secretions (200-500 µL)	Human	**SPE, column-switching system** [µBondapak with phenyl packing (150 mm x 3.9 mm)]	-	HPLC	µBondapak with phenyl packing (300 mm x 4.6 mm)	0.1 M ammonium acetate/ ACN (98:2, v/v)	UV (254 nm and 263 nm)	**LOQ:** 100 ng mL^{-1}	[116]
Cefuroxime	Sinonasal tissues	Human	-	NR	HPLC	NR	NR	NR	**LOQ:** 40 ng g^{-1}	[53]
Cefuroxime	Maxillary sinus mucosa (100-300 mg)	Human	**PP** [perchloric acid]	-	HPLC	Spherisorb ODS (125 mm x 4 mm, 1.5 µm)	4 mM perchloric acid /ACN (83:17, v/v)	UV (265 nm)	**Calibration range:** 300-50,000 ng mL^{-1} **LOQ:** 300 ng mL^{-1}	[76,112]
Ciclesonide desisobutyryl-ciclesonide	Nasal mucosa	Rabbit	Homogenization with ice-cold EtOH	Deuterium-labeled des-CIC	HPLC	Capcell pak UG120 C_{18} (50 mm x 2 mm, 3 µm)	ACN-EtOH(4:1)/5 mM ammonium acetate (65:35, v/v)	MS/MS	**Calibration range:** 0.5-1,000 ng mL^{-1} **LOQ** $_{(Ciclesonide)}$: 1 ng mL^{-1} **LOQ** $_{(des\text{-}CIC)}$: 0.5 ng mL^{-1}	[76]
Ciprofloxacin	Nasal secretions	Human	NR	-	HPLC	µBondapak C_{18} (150 mm x 4.6 mm, 5 µm)	0.025 M phosphoric acid, pH 3.0 adjusted with tetrabutyl-ammonium hydroxide /ACN (89:11, v/v)	Fluorescence $\lambda_{excitation}$ = 280 nm $\lambda_{emission}$ = 456 nm	NR	[95,117]
Danofloxacin	Nasal secretions	Calve	**PP** [ACN]	CP-71,755	HPLC	Octadecilsilane (15 cm, 5 µm)	0.01 mM phosphate buffer, pH 3.0 containing 0.008 M tetrabutylammonium sulphate and 0.005 M dibutylamine/ACN (89:11, v/v)	Fluorescence $\lambda_{excitation}$ = 280 nm $\lambda_{emission}$ = 440 nm	**LOD:** 10 ng mL^{-1}	[99]
Enoxacin	Nasal secretions	Human	NR	-	HPLC	Spherisorb ODS II C_{18} (5 µm)	ACN/0.1 M citric acid, 40 mM ammonium perchlorate and 5 mM tetrabutylammonium hydroxide (12-14:86-88, v/v)	UV (340 nm)	**Calibration range:** 78-5,000 ng mL^{-1}	[56]

Continued Table 2. Liquid chromatographic methods for quantitative determination of drugs in nasal and paranasal samples.

Drug	Sample (volume/weight)	Subject	Sample preparation	IS	Apparatus	Stationary phase	Mobile phase	Detection	Validation parameters	Ref.
Enrofloxacin	Nasal secretion (10 µL)	Pig	**SPE , column-switching system coupling RAM** [LiChrospher RP-18 ADS (25 mm x 4 mm, 25 µm)]	-	LC	Pursuit C_{18} (150 mm x 4.6 mm, 5 µm)	Gradient elution Phase A: 25 mM phosphate buffer, pH 3.0 adjusted with triethylamine Phase B: ACN	Fluorescence $\lambda_{excitation}$ = 278 nm $\lambda_{emission}$ = 445 nm	**Calibration range:** 90-15,000 ng mL^{-1} **LOQ:** 91.6 ng mL^{-1} **LOD:** 13.4 ng mL^{-1}	[44,55]
Fleroxacin	Maxillary sinus mucosa Nasal secretions	Human	**PP** [trichloroacetic acid]	AM 735	HPLC	Toyo Soda ODS-120 T (250 mm x 4.6 mm, 5 µm)	5 mM tetrabutyl-ammonium hydrogen sulphate/MeOH (72:28, v/v)	Fluorescence $\lambda_{excitation}$ = 290 nm $\lambda_{emission}$ = 450 nm	**LOQ $_{(sinus\ mucosa)}$:** 50 ng mL^{-1} **LOQ $_{(nasal\ secretions)}$:** 100 ng mL^{-1}	[118]
Fluticasone propionate	Nasal mucosa (3-25 mg)	Human	Microwave extraction	-	HPLC	NR	NR	MS/MS	NR	[75]
Gatifloxacin	Sinus fluid	Human	NR	-	HPLC	NR	NR	NR	**Calibration range:** 30-5,000 ng mL^{-1}	[92]
Levofloxacin	Sinus fluid	Human	NR	-	HPLC	NR	NR	NR	**Calibration range:** 20-4,000 ng mL^{-1}	[93]
Levofloxacin	Maxillary sinus mucosa (30-60 mg)	Human	**LLE** [dichloromethane]	Tinidazole	HPLC	C_{18} (5 µm)	Potassium phosphate/ACN, pH 2.6 (82:18, v/v)	UV (280 nm)	**LOQ:** 100 ng mL^{-1}	[49]
Ofloxacin	Nasal mucosa Septal cartilage Nasal conchae -spongy bone (100-500 mg)	Human	Nasal mucosa: homogenization with PBS . Bone/cartilage: pulverization in a mortar using liquid N_2; addition of PBS and centrifugation	-	HPLC	Hypersil ODS II (125 mm x 4.6 mm, 5 µm)	0.1 M phosphoric acid with 0.9% tributylamine/ ACN, pH 1.9 (90:10, v/v)	Fluorescence $\lambda_{excitation}$ = 290 nm $\lambda_{emission}$ = 480 nm	NR	[58]
Oxytretacycline	Nasal secretions (10 µL)	Pig	**PP** [perchloric acid 6%]	Metacycline	UHPLC	Acquity BEH C_{18} (50 mm x 2.1 mm, 1.7 µm)	Gradient elution Phase A: H_2O/ACN (95:5, v/v) Phase B: H_2O/ACN (5:95, v/v), both containing 1 mM oxalic acid and 0.1% formic acid	MS/MS	**Calibration range:** 42-19,000 ng mL^{-1} **LOQ:** 42 ng mL^{-1} **LOD:** 13 ng mL^{-1} **Recovery:** 99%	[45]
Moxifloxacin	Sinonasal mucosa	Human	NR	-	HPLC	Nucleosil 100 C_{18} (250mm x 4.6 mm, 5 µm)	Gradient elution Phase A: 0.01 M tetrabutylammonium sulphate/0.05 M sodium dihydrogen phosphate, pH 3.0 Phase B: ACN	Fluorescence $\lambda_{excitation}$ = 296 nm $\lambda_{emission}$ = 504 nm	**LOQ:** 500 ng mL^{-1}	[47,119]
Moxifloxacin	Maxillary sinus mucosa Ethmoid mucosa Nasal polyps (100 mg)	Human	**PP** [ACN/0.1 M aqueous phosphoric acid (1:1, v/v)]	-	HPLC	Nucleosil 100 C_{18} (250mm x 4.6 mm, 5 µm)	Gradient elution Phase A: 0.01 M tetrabutylammonium sulphate/0.05 M sodium dihydrogen phosphate, pH 3.0 Phase B: ACN	Fluorescence $\lambda_{excitation}$ = 296 nm $\lambda_{emission}$ = 504 nm	**LOQ:** 50 ng g^{-1} **Recovery:** 94-99%	[48,119]
Telithromycin	Nasal secretions (200 µL) Nasal mucosa (200-300 mg) Ethmoid bone (50-150 mg)	Human	Nasal secretions: **PP** [ACN] Nasal mucosa: **PP** [H_2O:ACN (30:70, v/v)] Ethmoid bone: **PP** [H_2O:ACN (30:70, v/v)]	HMR 3004	HPLC	Nucleodur CN (150 mm x 4.6 mm)	20 mM ammonium acetate/ ACN (40:60, v/v) + 0.20 ml glacial acetic acid	HMR 3004: UV (300 nm) Telithromycin: Fluorescence $\lambda_{excitation}$ = 263 nm $\lambda_{emission}$ = 460 nm	**LOQ $_{(nasal\ secretions)}$:** 10 ng mL^{-1} **LOQ $_{(nasal\ tissues)}$:** 30 ng g^{-1} **Recovery:** 80-95%	[46]
Theophylline	Olfactory mucosa (6-10 mg)	Mouse	**LLE** [dichloromethane]	-	HPLC	Zorbax Eclipse XDB-C_8 (150 mm x 4.6 mm, 5 µm)	Gradient elution Phase A: 0.05 M phosphate buffer, pH 2.3 Phase B: ACN	PDA (250nm)	NR	[120]
Zanamivir	Nasal secretions	Human	NR	-	HPLC	NR	NR	UV	**LOQ:** 5 ng mL^{-1}	[81]

ACN, acetonitrile; des-CIC, desisobutyryl-ciclesonide; EtOH, ethanol; IS, internal standard; H_2O, water; HPLC, high-pressure liquid chromatography; LC, liquid chromatography; LLE, liquid-liquid extraction; LOQ, limit of quantification; LOD, limit of detection; MeOH, methanol; MS, mass spectrometry; MS/MS, tandem mass spectrometry; N_2, nitrogen; PBS, phosphate buffered saline; PDA, photodiode array; PP, protein precipitation; SPE, solid-phase extraction; UHPLC, ultra-high pressure liquid chromatography; UV, ultraviolet. NR, not reported.

contribution of homogenization techniques for the assessment of drug concentrations in tissue samples, an inherent methodological limitation should be considered when the analytical results are interpreted. It is important to realize that tissue is not a uniform matrix and the measurement of drug concentrations using whole tissue homogenates represents an admixture of various compartmental fluids [43]. Indeed, the conventional homogenization procedures disrupt cell membranes and provide a suspension containing both intracellular and extracellular fluids which tend to inevitably underestimate the effective concentration of drugs in the tissues [53,123]. Assuming the low drug concentration levels generally present in nasal tissue samples, a particular attention should be given to this matter.

The choice of the sample pre-treatment procedure usually relies on analyte features, nature of the matrix, and detection method employed. Nasal/paranasal tissues and secretions are typically heterogeneous biological matrices that can be considered as protein rich samples; thereby, the implementation of an efficient sample clean-up procedure before the chromatographic analysis is mandatory. It is believed that unlike ultraviolet (UV) detection, the high selectivity of mass spectrometric and fluorescence detectors enables less extensive and elaborated sample pre-treatment approaches. Therefore, due to its simplicity and speed, PP has been widely used for the analysis of nasal/paranasal tissues and secretions by liquid-chromatography coupled with mass spectrometry or tandem mass spectrometry (LC-MS or LC-MS/MS) and HPLC coupled with fluorescence detection (HPLC-FD). Nevertheless, when high sensitivity is required, the presence of co-eluting endogenous substances and the occurrence of matrix effects may compromise the reliability of the analysis. Hence, regardless of the detection method involved, the removal of matrix interferences remains a fundamental issue. In general, to attain that goal, LLE and SPE procedures seem to be more appropriate than PP for nasal/paranasal samples pre-treatment, since they improve sensitivity by yielding cleaner extracts. For instance, in the method developed by Maes *et al.* [111] for the HPLC-MS/MS quantification of acyclovir in horse nasal fluid, a LLE with dichloromethane-isopropyl alcohol (50:50, v/v) was employed providing a lower limit of quantification of 2 ng mL^{-1} and a limit of detection of 0.05 ng mL^{-1}. Despite its advantages, conventional LLE and SPE methodologies are expensive, highly labour-intensive and time-consuming, involving numerous sample handling steps [110]. Owing to the small quantities of sample collected and consequently the sparse amount of drug generally present in nasal/paranasal specimens, the employment of extraction procedures that afford a minimum loss of sample during clean-up as well as high analyte recovery is needed. In this context, a column-switching technique coupling restricted access material (RAM) was developed by Bimazubute *et al*. [55] for the determination of enrofloxacin in pig nasal secretions. Another automated column-switching liquid chromatographic system was also reported to assess the penetration of ceftibuten in human nasal secretions [116]. On-line SPE techniques using different extraction supports such as RAM allow the direct injection of biological fluids, like serum or plasma, into the HPLC system; these procedures eliminate most of the time-consuming steps of manual sample preparation and, consequently, significant sample losses [37]. However, the direct injection of nasal secretions into chromatographic system is actually not technically feasible. Unlike plasma, the viscous character of nasal secretions often requires a previous sample dilution step with the corresponding washing liquid before HPLC injection [116]. Interestingly, in the technique developed by Bimazubute and collaborators [55], a 50-fold dilution of samples was performed not only for nasal secretions but also for plasma. In most cases, RAM sorbents function as a HPLC pre-column in combination with an analytical column and a column-switching system. RAMs are characterized by hydrophilic/hydrophobic, ion-exchange or size exclusion mechanisms which enable the removal of large molecules such as proteins and nucleic acids prior to chromatographic separation [37]. Generally, protein molecules quickly pass through the pre-column and the analytes of interest are efficiently retained on the adsorptive sites [37,124]. As a result, the target compounds are isolated and they can be promptly transferred onto an analytical column to proceed with the analyte separation and detection. During the development of a column-switching technique for on-line sample clean-up procedure, the parameters that could influence the extraction performance should be evaluated and optimized. Thus, among others, the SPE sorbent material, the composition of the washing liquid, the flow-rate, the times for the rotation of the switching valve and the transfer and separation steps might be accounted and investigated [55,125].

Last few years have witnessed extraordinary advances in the field of bioanalysis due to continuous technological progress targeting higher throughput and miniaturisation. In fact, several technological breakthroughs have been observed not only with the development of more powerful techniques and instrumentation for quantitative analysis of drugs and/or metabolites, but also with the emergence of novel sample preparation methodologies which have helped to improve extraction of the target

analytes from the biological matrices. Thus, numerous modern sample clean-up approaches such as RAM, liquid-liquid microextraction (LLME), solid-phase microextraction (SPME) molecularly imprinted polymers (MIP) and microextraction by packed sorbent (MEPS) have been successfully exploited [37]. Considering the characteristics of nasal specimens, particularly nasal secretions, MEPS assumes a special interest. Indeed, MEPS is a new sort of solid phase extraction that has been miniaturised to work with sample volumes as small as 10 µL and up to 250 µL [126]. The integration of the sorbent into the sampling syringe and the very small volumes required to elute the analytes (20-50 µL) allow MEPS to be used for direct injection into the chromatographic system. In general, the MEPS protocol involves few handling steps but a previous dilution of the sample is usually mandatory to reduce viscosity and prevent the blockage of MEPS cartridge. In comparison with SPE and LLE methods, MEPS technique is simple, fast, non-solvent consuming and provides similar degrees of selectivity and sensitivity [126]. Even though it has been mainly used for the extraction of drugs/metabolites from biological fluids such as blood, plasma or urine, MEPS seems to be also a very promising technique to be applied to nasal secretions after appropriate dilution.

2.3. Quantitative analysis in nasal and paranasal samples

2.3.1 Chromatography

The quantitative analysis of drugs and metabolites in samples obtained from nasal and paranasal regions faces an important and limiting issue related to the very small amount of sample available for bioanalysis. As a consequence, the target compounds are usually present in small quantities in the specimens which hamper and, even in some cases, may invalidate the assays. In fact, in the study carried out by Stoeckel and collaborators [79], a significant number of the sinus tissue samples analysed presented drug concentrations below the limit of quantification of the assay and, additionally, in some cases, insufficient amount of sample available for analysis did not allow a reliable determination. Likewise, during the pharmacokinetic study performed by Dinis *et al*. [53] about 25% of the sinus mucosa samples did not qualify for chromatographic analysis since the quantity collected was inadequate. Therefore, the development and validation of highly sensitive, selective and specific analytical techniques is mandatory to accomplish reliable, precise and accurate analysis of these low levels of analytes in restricted quantities of nasal/paranasal samples such as tissues or secretions. For instance, Bimazubute *et al*. [45] developed an ultra-high pressure liquid chromatography tandem mass spectrometry (UHPLC-MS/MS) method that enabled the quantification of oxytetracycline in barely 10 µL of nasal secretions of healthy pigs (Table 2). On the other hand, alternative microbiological bioassays were used to overcome the analytical inability of the chromatographic techniques to analyse small amounts of nasal/paranasal samples collected [84,113,127-129]. Although the quantity of sample needed to perform the analysis is reduced, they are time-consuming methodologies since the results can only be achieved after about 16-20 hours of incubation [129].

In the recent years, the technological progress and the continuous scientific advances have revolutionized the development of new analytical methodologies and instrumentation, providing more efficient and powerful techniques by decreasing the desired quantification levels and reducing the time of analysis to support the incessant requirements of the pharmaceutical industry and other research areas like toxicology. Currently, HPLC is still one of the most used analytical methodologies in bioanalysis. The liquid chromatography (LC) is able to separate and quantify low- and high-molecular-weight compounds from quite complex mixtures, using different mobile phases and chromatographic columns [110]. As it is clear from the Table 2, antibiotics are therapeutic agents most commonly analysed in nasal and paranasal matrices. In general, quantitative determinations of such drugs were achieved using reversed-phase C_{18} columns with either isocratic or gradient elution. In fact, HPLC takes a leading position on the bioanalytical field, presenting several advantages over other analytical methodologies like for example gas chromatography (GC). Unlike GC, HPLC allows for the analysis of non-volatile and thermolabile compounds and avoids the chemical derivatization procedures usually necessary for GC analysis. Thus, LC often requires less complicated sample preparation steps, covering a larger number of compounds susceptible to be analysed. Nevertheless, a few GC methods have been reported for the quantitative determination of drugs in nasal specimens. An example is the study performed by Hayden *et al.* [52] in which the nasal mucus concentrations of rimantadine and amantadine after derivatization with pentafluorobenzoyl chloride were assessed by GC with electron capture detection.

New developments enabled the introduction of reversed phase chromatography media with sub-2 µm particle size along with liquid handling systems that can operate such columns at much higher pressures, reaching values up to 1000 bar (or 15,000 psi) [124,130]. This technology, known as UHPLC, emerged as one of the latest innovations in liquid chromatography and presents significant theoretical advantages over

HPLC. Indeed, the use of stationary phases with small particle diameters (sub-2 µm of UHPLC-scale *versus* 5 µm of HPLC scale) in instrumentation with optimised characteristics, promotes significant gains in efficiency, sensitivity and speed for analytical applications when compared to the conventional HPLC [131]. Accordingly, this technology is a highly robust, reliable and reproducible system which allows the analysis of samples with increased resolution in shorter running times [131]. The technique developed by Bimazubute and collaborators [45] is a good example of the first UHPLC application for the analysis of nasal samples; using an analytical column packed with Acquity BEH C_{18} stationary phase, whose particle size was 1.7 µm, it was possible to perform the separation and quantification of oxytetracycline and metacycline (internal standard) in nasal secretions of pigs within a total run time of 2.5 min (Table 2). Most of the liquid chromatographic methods employed for the quantitative determination of drugs/metabolites in nasal and paranasal samples are summarized in Table 2. As was expected, LC using HPLC technology is still the analytical instrumentation mostly used for bioanalysis of small-molecule drugs in nasal and paranasal samples.

2.3.2. Detection

The hyphenation of chromatographic techniques to mass spectrometry (MS) systems has brought significant improvement in drug testing. In fact, LC-MS/MS is one of the most prevalent *hyphenated* techniques which, due to its inherent specificity, sensitivity and speed, has led to major breakthroughs in the field of quantitative bioanalysis since 1990s [124]. Thus, in addition to enabling the determination of extremely low levels of compounds in biological samples, it also provides structural information of the analytes affording its identification. The study performed by Maes and co-workers [111] imposed the need of using a hyphenated chromatographic technique in the quantification of an antiviral drug in nasal specimens. The purpose of their work was the determination of acyclovir in horse plasma and body fluids, such as nasal and cerebrospinal fluid; whereas in plasma the acyclovir concentrations were measured by means of a HPLC-FD method, in body fluids (nasal and cerebrospinal fluid) the research team resorted to LC-MS/MS since it allows a significant gain in sensitivity, being able to detect lower drug concentrations (ranging from 2 to 100 ng mL^{-1}) in small-volume samples (Table 2). Besides the above mentioned advantages, the universal character of LC-MS/MS allied to reduced sample pre-treatment requirements and shorter chromatographic run times [134,135], unequivocally renders this methodology a powerful tool in the development of any bioanalytical assay. Taking into account the demands of nasal and paranasal analysis, the potential of this methodology is of particular relevance. Hence, as mass spectrometers have become more easily accessible as well as easier to use, more laboratories have been able to purchase and exploit this technology. The latter can be ascertained by the reasonable number of published methods using LC-MS/MS for the quantification of drugs in nasal specimens, which constitute a valuable support to perform pharmacokinetic and toxicological studies (Table 2). However, mass spectrometers still remain highly costly equipments and the occurrence of matrix effects has been recognized as the main drawback that may compromise its wider use. In fact, the precision and accuracy of the analytical results could be affected by the presence of co-eluting endogenous matrix components present in the original biological sample that usually leads to ionization modifications of the target analytes, resulting in either ion suppression or enhancement phenomena [124,135,136]. Obviously, matrix effect has a greater impact on the low levels of the calibration curve than on the intermediate or higher values of the concentration range [124]. Thus, one of the major problems brought by this handicap regards the reduction of the sensitivity especially when the signal of the analyte is suppressed. Considering the small quantities of drugs generally present in the nasal samples analysed, this can be assumed as an essential and critical concern. For this reason, the influence of matrix effects must be studied and documented during the development process and validation of any LC-MS/MS-based analytical method, which can be attained by determining the peak responses ratio of extracted spiked samples and the corresponding neat drug solutions at the same concentration levels. Several strategies were studied and demonstrated to overcome matrix interferences [136], but this subject is beyond the scope of this review. The optimization of sample pre-treatment process, ionization method and mode, and composition of the mobile phase are some of the aspects that should be taken into account [124].

Bearing in mind that mass spectrometry is not often a promptly available detection technique, fluorescence and UV detection systems coupled to HPLC appear to be valuable alternatives considering their potential in terms of selectivity and sensitivity in addition to an easier access. Fluorescence detectors are also highly sensitive, extremely selective and specific since they can only be applied in the analysis of compounds with fluorescent properties. Therefore, the pharmacokinetic evaluation of antibiotic profiles used in the treatment of URTI took advantage of the fluorescent properties of

some molecules, such as fluoroquinolones, to employ HPLC-FD techniques in the measurement of these drug concentrations in nasal samples. Enrofloxacin [55], ciprofloxacin [95], ofloxacin [58], moxifloxacin [48], danofloxacin [99] and fleroxacin [118] are some examples of the fluorinated quinolones determined in nasal tissues or secretions of humans and animals (Table 2).

Although MS/MS and fluorescence provide better sensitivity and selectivity than UV detection, the latter is transversal to a large number of molecules, displaying a broader application in the bioanalytical field. In general, the sensitivity of the HPLC-UV method can be increased by performing the detection at lower UV wavelengths; however, the absorbance of co-eluting endogenous components of the biological matrices often impairs the accuracy and the specificity of the methodology. As a result, higher UV wavelengths are commonly applied and the sensitivity of the analysis is usually compromised. Even if mass spectrometry and fluorescence are by far the preferred methods of detection for quantitative analysis of samples with nasal and paranasal sources, several techniques employing UV detection have been reported, presumably because this detection system is practical and more readily available (Table 2). Alternative detectors applied in HPLC analysis include electron capture detection, which was used by Karma *et al*. [82] to determine azithromycin concentrations in sinus fluid and sinus mucosa samples of humans (Table 2). The employment of a radiometric HPLC system was also mentioned in literature to assess the toxicity of acetaminophen metabolites in the olfactory mucosa of mice [137,138]. In the latter, the detection of acetaminophen metabolites was carried out by radioactivity measurements with radiolabeled acetaminophen (^{14}C-acetaminophen) as substrate. Quantification of metabolites was accomplished by measuring radioactivity of individual peaks and the corresponding amounts were calculated based on percent recovery of total radioactivity [137,138].

2.3.3 Considerations on method validation

It is widely recognized that before a bioanalytical method can be implemented for routine use, it must first be validated to demonstrate its suitability for the intended analytical application, providing accurate, precise and reproducible data during sample analysis [38,139]. Effectively, all methodological stages should be investigated to determine the extent to which environment, matrix or procedural variables can affect the estimation of the analyte from the time of sample collection up to the interpretation of the obtained results [134]. Thus, bioanalytical method validation comprises all the procedures required to assure that a particular method used for the determination of analytes in a specific biological matrix, is reliable and reproducible [140]. It should also successfully meet or exceed the minimum standards recommended in the Food and Drug Administration (FDA) Guidance for Industry. According to FDA guidelines, the fundamental bioanalytical method validation parameters include accuracy, precision, linearity, selectivity, sensitivity, recovery and stability [141]. It was established that when a bioanalytical method is developed and implemented for the first time, a full validation is required; however, when a previously validated method is applied to a different matrix (*e.g*. plasma to urine), a partial validation may be sufficient. Rare matrices and limited quantities of sample, like nasal and paranasal specimens, usually impose the need of a partial validation. Such validation should at least provide data on the selectivity and sensitivity of the method since they are the key parameters of any bioanalytical assay. Moreover, the linearity, precision and accuracy of the measured concentrations should also be assessed. Taking into account the distinct enzymatic environments between different biological matrices, the stability of the analytes should also be studied.

For logical reasons, the most used biological matrices in bioanalysis are blood, serum, plasma and urine. Thus, it is not surprising that the majority of the analytical methods developed for the quantitative determination of drugs and/or metabolites have been performed in such matrices, and the application of these techniques to other specimens like saliva, cerebrospinal fluid, semen and even nasal/paranasal tissues and secretions is a reality. Indeed, it seems that a huge part of the pharmacokinetic studies performed in the nasal region were presented based on blood, plasma and urine analytical assays with minor modifications [47-49,56,79,95,113,118]. Nevertheless, the information about these alterations and its influence on the results has not always been clearly clarified. Ideally, the analytical techniques used for the measurement of drug concentrations in nasal samples, should be specifically developed and validated for this purpose. Unfortunately, the unavailability of sufficient amounts of blank nasal and paranasal specimens, either tissues or secretions, often precludes the preparation of calibration and quality controls (QC) in these matrices. As alternative different surrogate matrices like plasma [55] and even liver tissue [50] have been used to overcome this limitation. For instance, in the study performed by Fang *et al*. [50], human sinus mucosal tissue was employed for validation of the analytical method while, the calibration standards and QC samples were prepared in chicken liver during sample analysis owing to the limited supply of the actual

blank matrix. However, a distinct outcome regarding both sample recovery and matrix effects is a possible issue that could interfere with the analytical results and thus it should always be investigated in detail.

3. Conclusion

For many years, pharmacokinetic research was limited to drug concentration measurements in conventional biological samples such as blood, serum, plasma, urine and some tissue organs (*e.g.* liver and kidney). Taking into account that the primary target sites for most drugs are peripheral tissues, alternative specimens are often required to thoroughly investigate therapeutic efficacy and safety. Therefore, the importance of quantitative determinations of drugs and/or metabolites in both nasal/paranasal tissues and secretions is understandable due to the increasing prevalence of inflammatory and infectious diseases of the upper respiratory tract, as well as the increasing number of nasal formulations for topical, systemic or CNS drug delivery. Indeed, several pharmacokinetic studies using liquid chromatographic methods have been published to assess the penetration and exposure of drugs (*e.g.* antibiotics) in nasal compartments.

The collection of sufficient amounts of nasal/paranasal specimens to perform analytical measurements remains a great challenge. In fact, there are no ideal sampling methodologies and the standardization of the collecting procedures is still lacking. Sample preparation procedures should provide clean extracts, affording minimum sample losses and high analyte recoveries. LLE and SPE seem to be the most appropriate techniques for nasal/paranasal samples pre-treatment; however recent on-line extraction methodologies have emerged as promising sample clean-up approaches. Since the total quantity of drug in nasal/paranasal tissues or secretions is expected to be small; highly sensitive, selective and specific analytical techniques are required. LC-MS/MS has proved to be a suitable tool in this particular bioanalytical field.

In conclusion, only a few methods are developed and validated with the exclusive purpose of quantifying drugs and/or metabolites in nasal matrices. Effectively, pharmacokinetic studies are often being conducted using modified analytical methods initially developed for plasma or urine. Thus, additional efforts will be needed to exploit the potential of bioanalytical methods for quantitative measurements of drugs in nasal and paranasal specimens, providing valuable data to properly support either pharmacokinetic or toxicokinetic studies.

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References

[1] N.M. Veldhorst-Janssen, A.A. Fiddelers, P.H. van der Kuy, C. Neef, M.A. Marcus, Clin. Ther. 31, 2954 (2009)
[2] D.P. Wermeling, Neurotherapeutics 6, 352 (2009)
[3] A. Pires, A. Fortuna, G. Alves, A. Falcão, J. Pharm. Pharm. Sci. 12, 288 (2009)
[4] J.P. Wong *et al.*, Expert Rev. Respir. Med. 4, 171 (2010)
[5] C. Bitter, K. Suter-Zimmermann, C. Surber, Curr. Probl. Dermatol. 40, 20 (2011)
[6] L. Illum, J. Control. Release 87, 187 (2003)
[7] H.R. Costantino, L. Illum, G. Brandt, P.H. Johnson, S.C. Quay, Int. J. Pharm. 337, 1 (2007)
[8] R.J. Harvey, R.J. Schlosser, Otolaryngol. Clin. North Am. 42, 829 (2009)
[9] S. Türker, E. Onur, Y. Ozer, Pharm. World Sci. 26, 137 (2004)
[10] A. Minn *et al.*, J. Drug Target. 10, 285 (2002)
[11] Y.C. Wong, Z. Zuo, Pharm. Res. 27, 1208 (2010)
[12] M. Alagusundaram, Int. J. Res. Pharm. Sci. 1, 454 (2010)
[13] R.C. Dhakar, S.D. Maurya, V.K. Tilak, A.K. Gupta, G. Dangi, Int. Res. J. Pharm. 1, 29 (2010)
[14] M.M. Wen, Discov. Med. 11, 497 (2011)
[15] L. Illum, J. Pharm. Pharmacol. 56, 3 (2004)
[16] T.K Vyas, A. Shahiwala, S. Marathe, A. Misra, Curr. Drug Deliv. 2, 165 (2005)
[17] S.V. Dhuria, L.R. Hanson, W.H. 2nd Frey, J. Pharm. Sci. 99, 1654 (2010)
[18] S. Patel *et al.*, J. Drug Target. 19, 468 (2011)
[19] R.I. Henkin, Nat. Biotechnol. 29, 480 (2011)
[20] Y. Ozsoy, S. Gungor, E. Cevher, Molecules 14, 3754 (2009)
[21] V.S. Belgamwar *et al.*, Pharm. Dev. Technol. 14, 240 (2009)
[22] A.H. Elshafeey, E.R. Bendas, O.H. Mohamed, A.A.P.S. Pharm. Sci. Tech. 10, 361 (2009)
[23] J.C. Garcia-Rodriguez, I. Sosa-Teste, Scientific World Journal 9, 970 (2009)
[24] T.K. Kim *et al.*, Eur. J. Pharm. Sci. 38, 525 (2009)

[25] H.S. Mahajan, S. Gattani, Drug Deliv. 17, 19 (2009)
[26] F.C. Carvalho *et al.*, J. Pharm. Pharmacol. 62, 430 (2010)
[27] R. Jain *et al.*, Nanomedicine (Lond.) 5, 575 (2010)
[28] S. Khan, K. Patil, N. Bobade, P. Yeole, R. Gaikwad, J. Drug Target. 18, 223 (2010)
[29] R. Jain, V.B. Patravale, J. Biomed. Nanotechnol. 5, 62 (2009)
[30] D.J. Pillion, M.D. Fyrberg, E. Meezan, Int. J. Pharm. 388, 202 (2010)
[31] S. Gungor, A. Okyar, S. Erturk-Toker, G. Baktir, Y. Ozsoy, Drug Dev. Ind. Pharm. 36, 806 (2010)
[32] R. Jain, S. Nabar, P. Dandekar, V. Patravale, Pharm. Res. 27, 655 (2010)
[33] M. Kumar, A. Misra, K. Pathak, P.D.A. J. Pharm. Sci. Technol. 63, 501 (2009)
[34] S. Patil, A. Babbar, R. Mathur, A. Mishra, K. Sawant, J. Drug Target. 18, 321 (2010)
[35] N.R. Srinivas, Biomed. Chromatogr. 20, 383 (2006)
[36] N.R. Srinivas, Biomed. Chromatogr. 22, 235 (2008)
[37] P.L. Kole, G. Venkatesh, J. Kotecha, R. Sheshala, Biomed. Chromatogr. 25, 199 (2011)
[38] S. Pandey, P. Pandey, G. Tiwari, R. Tiwari, Pharm. Methods 1, 14 (2010)
[39] C.T. Viswanathan *et al.*, Pharm. Res. 24, 1962 (2007)
[40] M. Lim, M.J. Citardi, J.L. Leong, Am. J. Rhinol. 22, 381 (2008)
[41] P. Liu, M. Müller, H. Derendorf, Int. J. Antimicrob. Agents 19, 285 (2002)
[42] M. Müller, A. dela Peña, H. Derendorf, Antimicrob. Agents Chemother. 48, 1441 (2004)
[43] C.E. Edmiston *et al.*, Surg. Infect. 8, 179 (2007)
[44] M. Bimazubute *et al.*, J. Vet. Pharmacol. Ther. 33, 183 (2010)
[45] M.A. Bimazubute *et al.*, J. Chromatogr. B Analyt. Technol. Biomed. Life Sci. 877, 2349 (2009)
[46] T.S Kuehnel, C. Schurr, K. Lotter, F. Kees, J. Antimicrob. Chemother. 55, 591 (2005)
[47] P.B. Dinis, M.C. Monteiro, M.L. Martins, N. Silva, J.G. Morais, Ann. Otol. Rhinol. Laryngol. 113, 142 (2004)
[48] P. Gehanno *et al.*, J. Antimicrob. Chemother. 49, 821 (2002)
[49] F. Pea *et al.*, Pharmacol. Res. 55, 38 (2007)
[50] A.F. Fang *et al.*, Int. J. Antimicrob. Agents 34, 67 (2009)
[51] A. Ehnhage, M. Rautiainen, A.F. Fang, S.P. Sanchez. Int. J. Antimicrob. Agents 31, 561 (2008)
[52] F.G. Hayden, A. Minocha, D.A. Spyker, H.E. Hoffman, Antimicrob. Agents Chemother. 28, 216 (1985)
[53] P.B. Dinis, M.C. Monteiro, R. Lobato, M.L. Martins, A. Gomes, Laryngoscope 109, 1841 (1999)
[54] P.B. Dinis, M.C. Monteiro, M.L. Martins, N. Silva, A. Gomes, Laryngoscope 110, 1050 (2000)
[55] M.A. Bimazubute *et al.*, J. Chromatogr. A 1189, 456 (2008)
[56] U. Jaehde, F. Sörgel, K.G. Naber, J. Zürcher, W. Schunack, Antimicrob. Agents Chemother. 39, 2092 (1995)
[57] K. Takasaki, K. Enatsu, H. Kumagami, H. Takahashi, Int. J. Otolaryngol. 2009, article ID 495186 (2009), DOI: 10.1155/2009/495186
[58] P. Tolsdorff, Infection 21, 66 (1993)
[59] S. Zhang *et al.*, Chem. Res. Toxicol. 22, 949 (2009)
[60] J. Gu *et al.*, Mol. Pharmacol. 67, 623 (2005)
[61] G. Mainwaring, J.R. Foster, V. Lund, T. Green, Toxicology 158, 109 (2001)
[62] T. Green *et al.*, Chem. Biol. Interact. 137, 185 (2001)
[63] Z. Islam, C.J. Amuzie, J.R. Harkema, J.J. Pestka, Toxicol. Sci. 98, 526 (2007)
[64] B. Casado, Curr. Allergy Asthma Rep. 4, 224 (2004)
[65] Y.J. Zhang *et al.*, J. Pharm. Biomed. Anal. 50, 823 (2009)
[66] K. Go *et al.*, Auris Nasus Larynx 37, 427 (2010)
[67] B. Ghafouri, E. Kihlström, B. Ståhlbom, C. Tagesson, M. Lindahl, Biochem. Soc. Trans. 31, 810 (2003)
[68] H. Riechelmann *et al.*, Clin. Exp. Allergy 35, 1186 (2005)
[69] Y.S. Cho, S.Y. Park, C.K. Lee, B. Yoo, H.B. Moon, J. Allergy Clin. Immunol. 112, 695 (2003)
[70] C. Bachert, M.J. van Kempen, K. Höpken, G. Holtappels, M. Wagenmann, Eur. Arch. Otorhinolaryngol. 258, 406 (2001)
[71] S.J. Wilson, L. Lau, P.H. Howarth, Clin. Exp. Allergy 28, 220 (1998)
[72] R. Dahl, N. Mygind, Adv. Drug Deliv. Rev. 29, 3 (1998)
[73] S. Gizurarson, Acta Pharm. Nord. 2, 105 (1990)
[74] N. Jones, Adv. Drug Deliv. Rev. 51, 5 (2001)
[75] H. Petersen, A. Kullberg, S. Edsbäcker, L. Greiff, Br. J. Clin. Pharmacol. 51, 159 (2001)
[76] H. Sato *et al.*, B.M.C. Pharmacol. 7, 7 (2007)
[77] H. Sato *et al.*, Biopharm. Drug Dispos. 28, 59 (2007)
[78] Y. Zhang *et al.*, Biol. Pharm. Bull. 28, 2263 (2005)
[79] K. Stoeckel, M. Harell, M. Dan, Antimicrob. Agents Chemother. 40, 780 (1996)

[80] M.D. Alahmari, R.J. Sapsford, J.A. Wedzicha, J.R. Hurst, Transl. Res. 158, 163 (2011)
[81] D.P. Calfee, A.W. Peng, E.K. Hussey, M. Lobo, F.G. Hayden, Antivir. Ther. 4, 143 (1999)
[82] P. Karma, J. Pukander, M. Penttilä, Eur. J. Clin. Microbiol. Infect. Dis. 10, 856 (1991)
[83] M. Stenquist, L. Olen, M. Jannert, L. Näslund, M.L. Zeckel, Clin. Ther. 18, 273 (1996)
[84] G. Petrikkos, P. Goumas, E. Moschovakis, H. Giamarellou, Eur. J. Clin. Microbiol. Infect. Dis. 11, 828 (1992)
[85] J.B. Watelet, P. Gevaert, G. Holtappels, P. Van Cauwenberge, C. Bachert, Eur. Arch. Otorhinolaryngol. 261, 243 (2004)
[86] H. Riechelmann, T. Deutschle, E. Friemel, H.J. Gross, M. Bachem, Eur. Respir. J. 21, 600 (2003)
[87] F.X. Lü, R.E. Esch, J. Immunol. Methods 356, 6 (2010)
[88] L. Nikasinovic-Fournier *et al.*, J. Lab. Clin. Med. 139, 173 (2002)
[89] E.E. Walsh, A.R. Falsey, J. Infect. Dis. 179, 1268 (1999)
[90] L. Klimek, G. Rasp, Clin. Exp. Allergy 29, 367 (1999)
[91] T. Frischer, E. Baraldi, Am. J. Respir. Crit. Care Med. 162, 28 (2000)
[92] P.G. Ambrose *et al.*, Clin. Infect. Dis. 38, 1513 (2004)
[93] P.G. Ambrose *et al.*, Diagn. Microbiol. Infect. Dis. 61, 13 (2008)
[94] J.B. Anon *et al.*, Diagn. Microbiol. Infect. Dis. 57, 105 (2007)
[95] E.A. Piercy, R.E. Bawdon, P.A. Mackowiak, Antimicrob. Agents Chemother. 33, 1645 (1989)
[96] Y.F. Chung, M.D. Donovan, Int. J. Pharm. 128, 229 (1996)
[97] A.M. Sanico, G. Philip, G.K. Lai, A. Togias, J. Appl. Physiol. 86, 1202 (1999)
[98] B. Garré *et al.*, J. Vet. Pharmacol. Ther. 32, 207 (2009)
[99] C. Friis, Am. J. Vet. Res. 54, 1122 (1993)
[100] P. Mämmelä *et al.*, J. Environ. Monit. 4, 187 (2002)
[101] I. Ramis *et al.*, J. Chromatogr. 532, 217 (1990)
[102] E. Millqvist, E. Ternesten-Hasséus, A. Ståhl, M. Bende, Environ. Health Perspect. 113, 849 (2005)
[103] T. Heikkinen, M. Shenoy, R.M. Goldblum, T. Chonmaitree, Pediatr. Res. 45, 230 (1999)
[104] P.A. Dargaville, M. South, P. Vervaart, P.N. McDougall, Am. J. Respir. Crit. Care Med. 160, 778 (1999)
[105] H.C. Kaulbach, M.V. White, Y. Igarashi, B.K. Hahn, M.A. Kaliner, J. Allergy Clin. Immunol. 92, 457 (1993)
[106] C. Ward *et al.*, Thorax 48, 518 (1993)
[107] T.W. Marcy, W.W. Merrill, J.A. Rankin, H.Y. Reynolds, Am. Rev. Respir. Dis. 135, 1276 (1987)
[108] N. Kirschvink *et al.*, Vet. Res. 32, 145 (2001)
[109] A. Virolainen *et al.*, J. Clin. Microbiol. 31, 1382 (1993)
[110] L. Nováková, H. Vlčková. Anal. Chim. Acta 656, 8 (2009)
[111] A. Maes *et al.*, Biomed. Chromatogr. 23, 132 (2009)
[112] R. Wyss, F. Bucheli, J. Chromatogr. 430, 81 (1988)
[113] P. Cherrier *et al.*, Eur. J. Clin. Microbiol. Infect. Dis. 12, 211 (1993)
[114] F. Kees, W. Raasch, M. Steger, H. Grobecker, J. Chromatogr. 525, 484 (1990)
[115] F. Camus, A. Deslandes, L. Harcouet, R. Farinotti, J. Chromatogr. B Biomed. Appl. 656, 383 (1994)
[116] P. Krumpe, C.C. Lin, E. Radwanski, M.N. Cayen, M.B. Affrime, Chest 116, 369 (1999)
[117] B. Joos *et al.*, Antimicrob. Agents Chemother. 27, 353 (1985)
[118] E. Weidekamm, R. Portmann, Am. J. Med. 94, 75 (1993)
[119] H. Stass, A. Dalhoff, J. Chromatogr. B Biomed. Sci Appl. 702, 163 (1997)
[120] V. Gudziol, J. Pietsch, M. Witt, T. Hummel, Eur. Arch. Otorhinolaryngol. 267, 239 (2010)
[121] R.J. England, J.J. Homer, L.C. Knight, S.R. Ell, Clin. Otolaryngol. Allied Sci. 24, 67 (1999)
[122] M.I. Ugwoke, N. Verbeke, R. Kinget, J. Pharm. Pharmacol. 53, 3 (2001)
[123] D.E. Nix, S.D. Goodwin, C.A. Peloquin, D.L. Rotella, J.J. Schentag, Antimicrob. Agents Chemother. 35, 1947 (1991)
[124] R.N. Xu, L. Fan, M.J. Rieser, T.A. El-Shourbagy, J. Pharm. Biomed. Anal. 44, 342 (2007)
[125] P. Campíns-Falcó, R. Herráez-Hernández, A. Sevillano-Cabeza, J. Chromatogr. 619, 177 (1993)
[126] M. Abdel-Rehim, Anal. Chim. Acta 701, 119 (2011)
[127] F. Fraschini *et al.*, J. Antimicrob. Chemother. 27, 61 (1991)
[128] G. Kment, A. Georgopoulos, W. Ridl, J. Mühlbacher, Eur. Arch. Otorhinolaryngol. 252, 236 (1995)
[129] S.E. Holm, A.M. Bergholm, C. Ekedahl, K. Lundin, J. Laryngol. Otol. 97, 59 (1983)
[130] D. Sharma, R. Mittal, A. Gupta, K. Singh, A. Nair,

J. Pharmaceut. Biomed. Sci. 7, 1 (2010)
[131] L. Nováková, L. Matysová, P. Solich, Talanta 68, 908 (2006)
[132] M. Vogeser, C. Seger, Clin. Biochem. 41, 649 (2008)
[133] D.M. Müller, K.M. Rentsch, Anal. Bioanal. Chem. 398, 2573 (2010)
[134] P. Wall, B. Kumar, A. Bhandari, A.K. Rai, A.Wal, J. Pharmaceut. Sci. Technol. 2, 333 (2010)
[135] M. Jemal, Biomed. Chromatogr. 14, 422 (2000)
[136] B.K. Matuszewski, M.L. Constanzer, C.M. Chavez-Eng, Anal. Chem. 75, 3019 (2003)
[137] J. Gu *et al.*, J. Pharmacol. Exp. Ther. 285, 1287 (1998)
[138] M.B. Genter *et al.*, Biochem. Pharmacol. 55, 1819 (1998)
[139] S. Bansal, A. DeStefano, A.A.P.S. J. 9, 109 (2007)
[140] V.P. Shah *et al.*, Pharm. Res. 17, 1551 (2000)
[141] Guidance for Industry: Bioanalytical method validation (US Food and Drug Administration, USA, 2001) http://www.fda.gov/downloads/Drugs/GuidanceComplianceRegulatoryInformation/Guidances/UCM070107.pdf