THEORETICAL AND APPLIED PERSPECTIVES IN HEALTH SCIENCES

Editor: Prof. Dr. Gönül GÜROL ÇİFTÇİ

DUVAR

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Tel: 0 232 484 88 68

www.duvaryayinlari.com
duvarkitabevi@gmail.com

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Chapter 1

Bartonella henselae and Zoonotic Significance

Derya KARATAŞ YENİ[1], Muhammed Can GÖKMEN[2], Asma ASHRAF[3]

Introduction

Infections that are capable of being transmitted between animals and humans, with or without the involvement of vectors, are classified as zoonotic infections. It is estimated that there are approximately 1,500 pathogens that have the potential to infect humans, of which 61% are responsible for the emergence of zoonotic diseases (Rahman et al., 2020; Taylor et al., 2001). Today, the incidence of zoonotic diseases has increased due to changes caused by global warming, unconscious and uncontrolled antibiotic use, more intensive animal breeding and increased pet feeding at home (Cantas & Suer, 2014). The 'One Health' approach, a concept with a history dating back to the ancient times of Hippocrates, is predicated on the unique dynamic interaction between humans, animals and pathogens sharing the same environment (Calistri et al., 2013). The transmission of bacterial pathogens causing zoonotic diseases from animals to humans can occur via bites and scratches, through animal foods, or by direct contact with individuals working in the livestock sector (Glaser et al., 1994; Zambori et al., 2012).

This phenomenon has been particularly marked in the period following the emergence of the novel Coronavirus (SARS-CoV-2) pandemic (Kaymaz, 2021). This rapprochement between humans and animals has many positive aspects, as well as some inevitable negative consequences. The cohabitation of humans and pets has led to an observed escalation in the prevalence of zoonotic diseases.

[1] Associate Professor Doctor, Necmettin Erbakan University Faculty of Veterinary Medicine, 0000-0001-7261-1394
[2] Research Assistant, Necmettin Erbakan University Faculty of Veterinary Medicine, 0009-0003-6385-5495
[3] Professor Doctor, Government College University Faisalabad Department of Zoology, 0000-0001-7026-670X

Some of these zoonotic diseases have been reported to cause infections in both animals and humans with various symptoms (Damborg et al., 2016; Halsby et al., 2014; Paul et al., 2010). Despite the fact that certain zoonotic infections can be prevented through vaccination, it should be noted that no such vaccine is currently available for some infections (Monath, 2013). Since some zoonotic infections are asymptomatic in animals, there is no chance of protection in advance (Chomel, 2014). Cat Scratch Disease (CSD) is an example of such an infection. *B. henselae*, a bacterium that is capable of surviving within the erythrocytes of cats, is typically carried asymptomatically within the population of cats. However, it has been demonstrated that the bacterium can cause infection in kittens and cats with compromised immune systems (Kordick & Breitschwerdt, 1995).

The following *Bartonella* spp. have been identified as playing a primary role in the etiology of the infection: *Bartonella henselae*, *Bartonella clarridgeiae*, *Bartonella koehlerae*, *Bartonella quintana* and *Bartonella doshiae (Lamas et al., 2008)*. Felines (*Felis catus*) act as vectors for this zoonotic bacterial pathogen, transmitting it to humans through biting or scratching. Moreover, it has been documented that the infection can be transmitted from cats to humans through contact with cat saliva on damaged skin or sclera (Cantas & Suer, 2014). Consequently, the presence of *B. henselae* cannot be diagnosed during routine veterinary examinations. In humans, CSD has been observed to present with symptoms including fever, lymphadenopathy and anaemia. The disease is known to cause systemic infections in individuals with compromised immune systems. In human medicine, the presence of *B. henselae* may be overlooked in routine examinations (Cheslock & Embers, 2019).

Etiology

Cat scratch disease (CSD) is a clinical syndrome that has been reported in humans for over a century. However, it was not until 1992 that the infection was identified as being transmitted by cat scratches or bites, and that the etiological agent was identified as *Bartonella henselae (Elder et al., 2011)*. *B. henselae*, the primary causative agent of cat scratch disease, is a Gram-negative bacterium in the genus *Bartonella* of the family *Bartonellaceae*, class *Proteobacteria*. The causative agent is bacillus-shaped and measures 0.3 to 0.6 x 1.0 to 1.7 μm. Pleomorphic, unencapsulated and spineless. *B. henselae* has no flagella but the organism can move due to the presence of pili (Benson et al., 1986; Jaffe et al., 2018). *B. henselae* has been found to be negative for catalase, oxidase, urease and nitrate reductase. The causative agent is found to be highly haemine dependent. Consequently, the utilisation of enriched blood-containing media is imperative

for its isolation. *B. henselae* demonstrates optimal growth at 37 °C in the presence of 5-10% CO_2. The resultant colonies are white, dry, and fluffy, and demonstrate a penchant for engraving the medium (Diddi et al., 2013).

Although studies on the pathogenesis of *B. henselae* are limited, three virulence factors have been identified to date. These are *Bartonella* adhesinA (BadA), VirB/VirD4 Type 4 secretion system and Trw type 4 secretion system (Franz & Kempf, 2011; Seubert et al., 2003). BadA provides bacterial autoagglutination, adhesion to host cells and binding to extracellular matrix proteins (Kaiser et al., 2008). The VirB/VirD4 type 4 secretion system is responsible for the translocation of *Bartonella* effector proteins (Beps) into host endothelial cells. This process subsequently triggers a number of host responses that may be crucial for bacterial colonisation of the endothelium (Schröder & Dehio, 2005). The Trw type IV secretion system is a necessary factor for the intracellular colonisation of the agent in erythrocytes. In addition to this function, the Trw system has been demonstrated to mediate the host specificity of infections occurring in erythrocytes (Vayssier-Taussat et al., 2010). Furthermore, the ability of *B. henselae* to form biofilms has been demonstrated in vitro. Biofilm formation of the bacterium causes chronic and relapsing infections in the vertebrate host. In addition, the biofilm formation of the agent in the Ctenocephalides felis vector is also important in its transmission (Okaro et al., 2021).

Epidemiology

Cat Scratch Disease is widespread all over the world (Gagliardi et al., 2014). The causative agent of the disease, *B. henselae*, is the most common species of *Bartonella* spp. causing human infection (Jackson et al., 1993). It has been documented that the seroprevalence of antibodies against *B. henselae* and *B. henselae* bacteremia is elevated in human populations, particularly in regions characterised by a hot and humid climate (Dalton et al., 1995). The prevalence of *Bartonella* in cats has been reported to range from 4% to 70% using blood culture methods, and the seroprevalence of antibodies to Bartonella in cats ranges from 0% to 80% (Köseoğlu et al., 2022; Pons et al., 2005).

The primary reservoir of *B. henselae* is cats, but humans and dogs can also be hosts for the agent (Deng et al., 2012; Kim et al., 2009; Minnick & Barbian, 1997). Although 50% of cats carry the causative agent, most are asymptomatic (Zangwill et al., 1993). Vectors such as fleas, lice and ticks play a role in the transmission of the infection between cats (Diaz et al., 2012; Yeni, 2021). *Ctenocephalides felis*, or the cat flea, has been identified as a significant vector in the horizontal transmission of the disease between cats. Furthermore, *Ixodes*

ricinus ticks have been documented as a vector responsible for the transmission of *B. henselae* among cats (Cotté et al., 2008). Direct and frequent contact of cats and dogs infected with *B. henselae* with humans poses a potential risk for the transmission of the causative agent (Ihler, 1996). The transmission of infection from cats to humans is facilitated by biting and clawing. Furthermore, the agent can also be transmitted to humans through tick bites (Klotz et al., 2011; Massei et al., 2005). It has been documented that certain occupational categories, such as veterinarians who engage in frequent direct contact with animals, are predisposed to elevated levels of exposure to *B. henselae* (Sepúlveda-García et al., 2023).

B. henselae Infections

Felines act as natural reservoirs for *B. henselae*, often developing asymptomatic intraerythrocytic bacteraemia, which can persist for extended periods ranging from months to years (Kordick & Breitschwerdt, 1995). Although CSD is asymptomatic in the majority of cats, it can cause fever, anaemia, lymphadenomegaly, cardiac and renal failure, lethargy and neurological symptoms in kittens and immunosuppressed animals (Abbott et al., 1997).

In human subjects, the infection leads to lymphadenopathy syndrome accompanied by fever. The onset of the disease is characterised by the appearance of an erythematous papule at the site of inoculation. The papule manifests between three and ten days following inoculation and evolves through erythematous, vesicular, and papular crusted stages. The lesion persists for 1 to 3 weeks. The occurrence of regional lymphadenopathy manifests within a timeframe of 1 to 3 weeks following the initial inoculation. Systemic infection manifests as a mild condition in the majority of patients, presenting with symptoms such as fever, generalised aches, malaise, anorexia, nausea and abdominal pain (Florin et al., 2008). The bacterium is known to cause serious systemic infections, especially in individuals with compromised immune systems, and can also result in neurological symptoms. Untreated Bartonellosis has been shown to have severe consequences (Cheslock & Embers, 2019; Marra, 1995).

Diagnosis

The causative agent is highly haemine dependent. Consequently, the isolation of the causative agent necessitates the use of enriched blood-containing media. *B. henselae* demonstrates optimal growth at 37 ^{o}C in the presence of 5-10% CO_2. The colonies formed on the medium are white, dry, fluffy, and engrave the medium (Diddi et al., 2013). The time frame for visible colony formation is 45 days for the first isolation and 5-15 days for passaging. The sluggish growth rate

of these bacteria renders conventional biochemical identification methods impractical (La Scola & Raoult, 1999; Maurin et al., 1994). The diagnosis of *B. henselae* infection is facilitated by serological or molecular-based methods. Serological tests are suitable for confirming current or past infection in vivo. These tests have been shown to exhibit high sensitivity but low specificity (La Scola & Raoult, 1996). Among molecular methods, PCR is distinguished by its rapid, sensitive and specific capacity to detect the organism directly from clinical samples. For this purpose, the *gltA* gene region of the causative agent is utilised (Birtles & Raoult, 1996). It has also been reported that whole cell fatty acid (CFA) analysis can be used for identification between species (Clarridge 3rd et al., 1995).

Conventional bacteriological methods for the diagnosis of infection in cats are often impractical. The fact that *B. henselae* is asymptomatic in the majority of cats means that its diagnosis is not possible during routine examinations. In humans, laboratory findings are usually non-specific, with the exception of tests aimed at the identification of CTD. Diagnosis is typically based on anamnesis and clinical findings (Kozubaev et al., 2024). Infection may result in a normal or slightly elevated white blood cell count and a normal, elevated or decreased platelet count in the blood picture. In the context of diagnostic tests for infection, the indirect fluorescence test (IFA) and the enzyme immunoassay (EIA) tests are frequently utilised as serological methods, while the polymerase chain reaction (PCR) is commonly employed as a molecular method. However, it is not routinely preferred due to the extended time required for culture isolation (Florin et al., 2008). The aforementioned infection has been observed to result in basilar angiomatosis, basilar pelliosis and neurological syndromes in immunocompromised individuals (Çelebi, 2008).

Treatment and Prevention

Antibiotics such as amoxicillin and amoxicillin-clavulanic acid are generally used in cats infected with *B. henselae*. However, given the intracellular nature of the causative agent, the efficacy of antibiotic treatment may be constrained (Nivy et al., 2022). Nevertheless, the administration of antibiotics to healthy cats with bacteraemia, residing in the same household with immunocompromised adults or young children, is strongly advised. In human subjects, lesions are usually limited in cases of CTD infection. However, the use of antibiotics may be necessary in certain people with systemic infection. These include azithromycin, rifampin, ciprofloxacin, trimethoprim/sulfamethoxazole and gentamicin (Margileth, 1992; Rolain et al., 2004) . There exist general treatment recommendations for feline and canine Bartonellosis, which are based on the studies carried out to date. In

order to minimise drug resistance to the causative agent and to allow the disease to be cured (4-6 weeks), it is recommended (Okaro et al., 2021).

Conclusion

Cat Scratch Disease (CSD), a zoonotic infection caused by *B. henselae*, is a serious public health concern that should be given greater societal awareness. The consequences of failing to diagnose this condition can be grave.

The transmission routes of *Bartonella* spp. are not yet fully understood. It is hypothesised that various rodents and vectors are involved in the spread of the disease. In order to prevent the transmission of the pathogen between animals and from animals to humans, and to apply optimal measures, it is necessary to focus on the sources of disease transmission in a wide framework. A further finding of the current literature review is that the seroprevalence rates of *Bartonella* spp. in some settlements worldwide are not fully compatible with the low number of clinical cases. This incongruity can be attributed to the potential for *Bartonella* infections to evade detection during diagnostic procedures.

This zoonotic agent, which can pose a significant threat to public health, is of paramount importance in both veterinary and medical medicine. Consequently, there is a necessity for research activities to be carried out within a single health context. The environment, transmission routes, and the factors that patients are exposed to in their social lives should also be examined, and anamnesis should be handled as a whole. Vaccination studies are also required to protect both animal and human health.

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Chapter 2

Electroencephalography and Differential Diagnosis of Epileptic Waves

Hasan YAŞAR[1]

Electroencephalography (EEG)

EEG is a recording of bioelectrical activity originating from the cerebral cortex. Potential differences between electrodes are measured. In a relaxed state of wakefulness with eyes closed, sinusoidal waves with a frequency of 8–13 Hz in the occipital lobe are called alpha waves (1). Alpha rhythm is most pronounced in the occipital region and shifts anteriorly during sleep. In human development, the 8 Hz alpha frequency normally occurs around age 3. Frequencies higher than 13 Hz are called beta rhythms. Beta rhythms usually occur with the use of benzodiazepines, barbiturates, and chloral hydrate. It may decrease with eye opening, alertness, or movement. It can be observed as bifrontal. Beta activity normally increases drowsiness and light sleep (2). Theta rhythms (4 -7 Hz) can occur normally at rest. Hyperventilation can produce normal variations of the EEG and increased delta activity (including intermittent rhythmic delta and theta activity). That condition ends within 2 minutes after stopping hyperventilation (3). The information provided by routine EEG is limited. To identify epileptiform activity on surface EEG, a discharge in a 6 cm^2 tissue area is required. It explains why most simple partial seizures are not recorded on surface EEG (4). For example, epileptiform activity spreading from the mesial temporal region is often not detected on surface EEG (5). The duration of the recording is also important. The short duration of the recordings usually does not allow the identification of interictal epileptiform activity. The EEG should be examined with the recommended filter setting (0.3 Hz LFF and 70 Hz HFF) (2).

Epileptic Waves

Epileptic waves in EEG are spike and sharp waves. First of all, some principles should be taken into consideration when reading EEG to prevent

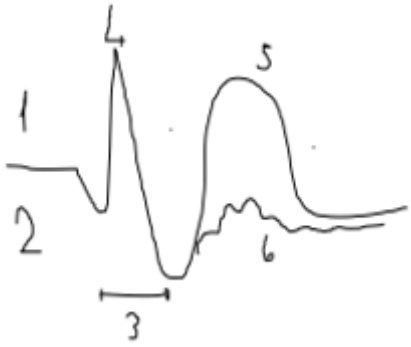
[1] Assoc. Prof. Dr. Trabzon Kanuni Training and Research Hospital, Department of Neurology

misdiagnosis. These are; Unless there are one or more valid reasons to suspect otherwise, every wave that appears spiky is an artifact! We should not forget the rule.

1- Brain-derived spikes and sharp waves always occupy an identifiable electric field on the scalp and should always be seen at 2 or more nearby electrode sites.
2- Clinically significant spikes and sharp waves are almost always initially of superficial negative polarity, or at least the sharpest or highest voltage component of the wave is usually superficial negative. It is asymmetrical. It is bi- or triphasic waves and has slow waves following them. It has a different appearance from the background activity.
3- Most spike or sharp wave discharges of clinical significance are followed by a slow wave or train of slow deflections. If there is no slow trailing wave, one should be more suspicious of artifact or a sudden change in the voltage of physiological background rhythms. Shorter or longer wave durations occur in contrast to ongoing background activity. Background deterioration with epileptic discharge should occur.
4- Spikes and sharp waves that can be explained by changes in background activity are ignored.
5- Physiological spikes and sharp waves that occur during sleep should be well known.

Spike waves usually have a period between 20 and 70 milliseconds, while a sharp wave with a negative surface component usually has a period between 70 and 200 milliseconds. These criteria alone are not sufficient to distinguish epileptic discharges from other waveforms. Thus, the difference between spikes and sharp waves is based only on duration, but their clinical significance is the same. The function of the bipolar montage is to compare 2 active electrode points, while the reference montage compares the active and common electrode point. When the voltage of the first electrode is more electronegative than the voltage of the second electrode, the waveform deflection is upward. Similarly, if the voltage of the second electrode is more negative than the voltage of the first electrode, the waveform will be deflected downward. Normal and abnormal phase reversal can occur as electronegative events. Rarely, electropositive phase reversals with abnormal spikes and sharp waves may occur. Phase reversals determine regions of maximum electronegativity (and electropositivity). Therefore, the occurrence of phase reversals may indicate a normal finding and does not indicate an abnormality in the EEG. It is known that phase reversals are found in almost all normal sleep recordings. Phase reversals may also occur in

normal waveforms and artifacts. When reading EEG, it is always important to note whether phase reversals represent a physiological field. If it does not represent a physiological field, it should not be interpreted as epileptic (3, 6, 7). The sharp component of epileptiform discharges is typically negative. Positive spikes and sharps are rare and may be seen in patients undergoing resective brain surgery. Regional slowing of EEG activity is not specific for epilepsy and therefore does not support a diagnosis of epilepsy (8, 9). Rhythmic activity with phase reversals isolated to a single electrode without a field should be interpreted as artifact until proven otherwise. Double and triple phase reversals should also raise suspicion of artifact (10).



1: Negative direction, above the isoelectric line

2: Positive direction, below the isoelectric line

3: Duration, spike(20ms-70ms), sharp(70ms-200ms)

4: Pointed top part. It is seen asymmetrically in spike and sharps. Sudden rise, slow decrease.

5: Slow wave, follows the spike and sharps.

6: The isoelectric line is disrupted differently from what happens in physiological waves, if the slow wave does not follow.

Interictal epileptiform activity includes spikes, sharp waves, and paroxysmal fast activity and their combinations with slow waves, such as spike-wave complexes (spike followed by slow wave) and polyspike-wave complexes (multiple spikes followed by slow wave). Focal interictal epileptiform activity can be activated by hyperventilation and sleep deprivation, but is very rarely activated by photic stimulation (4). Indications for video-EEG monitoring include the differential diagnosis of paroxysmal events such as epileptic seizures, organic nonepileptic seizures, and psychogenic nonepileptic seizures (11).

Artifacts

Findings that raise suspicion of artifact in EEG:

1-If the activity or waveform is limited to only 1 channel, it is an artifact until proven otherwise.

2-Activity observed in more than one non-adjacent region is considered as an artefact.

3-Complex waveforms with alternating double and triple phase reversals; represent a field that does not originate from the brain generator.

4. Atypical generalized waveforms suggest the potential for an equipment artifact involving all channels.

5. Very high or very slow frequencies <1 Hz or >70 Hz; Most of the activity in the brain is between 1 and 35 Hz (12).

Complex physiological movements such as eye movements, glossokinetic movements, head movements, pulse, tremor, and myoclonus, and psychogenic nonepileptic attacks are common sources of physiological waves on the EEG that may resemble seizures (10). Usually muscle-related "spikes" are waves that occur on the temporal and frontalis muscles and can be confused with abnormal epileptic discharges (3).

Benign Variants

Benign variants usually occur during drowsiness and light sleep and do not disrupt the background rhythm (13). It does not indicate specific pathological conditions. They are more common in the temporal lobes and may mimic epileptiform discharges. They are rarely seen while awake or in deep sleep (3).

Mu rhythm: The mu rhythm is a common arcuate alpha frequency seen in approximately 25% of normal EEGs and reflects the resting state of the rolandic cortex. It is suppressed by the movement of the opposite extremity and is easily recognized. Although it is an alpha variant, it is not blocked by eye opening (2, 14).

14 and 6 positive waves: It is seen as a single phase in the temporal regions in adolescents and adults during wakefulness and sleep stages 1 and 2 at frequencies of 14 and 6 Hz. Its duration is less than 1 second. It disappears in deeper levels of sleep. This pattern is most common between the ages of 8 and 14 years, decreases in adolescence, and is rare in early adulthood (3). It consists of rhythmic series of spike discharges at 6 or 14 Hz. Best distinguished with the contralateral ear reference electrode (15). It consists of surface positive spiky and surface negative round arch wave trains (16).

6 hz spike and wave: It can be seen in all regions at a frequency of 5-7 Hz, but is most common in the occipital and frontal regions. It is biphasic. It has a

small spike and a wide wave. It is also seen in sleep stage 1 in adolescents and adults, it has a duration of less than 1 second, it is also called ‘Phantom’ spike and wave (3). The phantom spike-waves are more abundant on Cz-Pz and are parietal dominant. Phantom spike-and-wave bursts are said to be "phantom" because the amplitude of the spike wave is low compared to the slow wave component that follows it, and the slow wave is more widely dispersed (17). A distinctive feature is their presence during relaxed wakefulness and light sleep and their disappearance at deeper levels of sleep (16). They appear to be more associated with seizures when they occur with high-amplitude spikes and occur at a frequency of less than 6 Hz or when they occur during wakefulness and persist into slow-wave sleep (2).

***Wicket spikes*:** It occurs at a rate of 6-12 Hz in adults, in the temporal region, during wakefulness or sleep stage 1, and lasts 0.5-2 seconds. It has characteristics similar to sleep spindles (8). Most patterns mistakenly considered epileptic are benign temporal sharp transients or wicket spike waves (13). There is no slow wave after the waves of wickets. It does not disturb the background activity. It is most prominent during drowsiness and light sleep, but can also occur during wakefulness or during arousal bursts (3). It occurs in adults over 30 years of age and occurs in the 6 to 11 Hz band.

***Small sharp spikes=Benign epileptiform transients of sleep (BETS)=Benign sporadic sleep spikes (BSSS)*:** In adults, it is mostly seen in the frontal regions as a single discharge at rest and in sleep stages 1 and 2 (17). It can be distinguished from epileptiform spikes and slow wave discharges because of the absence of background activity disturbance, the absence of associated focal abnormalities, bilateral occurrence, and disappearance in deeper sleep levels (16).

Sreda (Subclinical rhythmic elektographic discharge of adults): It starts and ends suddenly with a frequency of 5-6 Hz. It is seen in wakefulness, hyperventilation and sleep stage 1. It occurs in the elderly and lasts 40-80 seconds (8). It is the rarest of the benign variants, with an incidence of 0.05% and often occurs during wakefulness. It is common in the parietal-posterior-temporal regions. The only known trigger is hyperventilation. It is distinguished from the ictal pattern by the absence of temporal-spatial changes and preservation of the alpha rhythm. During pattern disappearance, baseline EEG activity is rapidly restored without post-seizure slowing (3). Sreda is performed while the patient is awake so that the mental state can be understood while the EEG is being recorded. A patient with the sreda pattern will not experience a change in level of consciousness (16). Unlike most benign variants that occur more in younger age groups and during the transition to sleep, sreda occurs in the over 50 age population and during wakefulness (2).

Lambda waves and Positive occipital sharp transients of sleep (POST): These two benign patterns are grouped together because they have similar appearance and location. Lambda waves have a triangular shape and appear in the occipital regions during active visual scanning of complex scenes or during reading. They can be symmetrical or asymmetrical and are usually diminished when looking at something lacking in detail (18). It occurs when the eyes are open. It disappears when the eyes are closed and during sleep. It is limited to the occipital regions (17). It is best observed in young adults (2). Post are positive sharp transients occurring in occipital regions during NREM sleep, which are associated with other sleep EEG figures (K-complex, spindles) (14). Post represents stage 1 sleep (2). Hyperventilation-induced slowing may be mistakenly interpreted as epileptiform (18). Normal variants tend to be more prevalent in the early stages of sleep and are therefore more likely to be considered abnormal if a similar waveform persists in the deeper stages of sleep (N3) (3).

Nonconvulsive status epilepticus (NCSE)

EEG is of great importance in detecting and distinguishing NCSE (19). Any seizure pattern showing epileptiform discharges faster than 2.5 Hz in a comatose patient reflects nonconvulsive seizures or NCSE and should be treated. There is no universally accepted definition of NCSE. Epileptiform EEG patterns, such as generalized periodic spikes, may be seen in patients in deep coma, and there is ongoing debate about the relationship between these patterns and NCSE. EEG patterns in coma are related to coma depth and clinical examination. Periodic lateralized epileptiform discharges (PLEDs) or bilateral independent periodic epileptiform discharges (BIPEDs) occurring in comatose patients provide limited information in the diagnosis of NCSE in coma. In a comatose patient, epileptic etiology is definitively suspected by the presence of evolving continuous or intermittent epileptiform discharges or rhythmic slow wave patterns on the EEG (8,9). PLEDs are transient phenomena that last several weeks (20).

Sleep EEG

Sleep spindles appear periodically at a frequency of 12–15 Hz in all stages of sleep (1). Sleep stage 1 is defined by the presence of vertex waves, which are sharp biphasic transitions of 200 milliseconds, usually with maximum negativity at the vertex (Cz) electrode. During sleep, vertex waves and positive occipital sharp transitions of sleep may be mistakenly identified as sharp waves. This is especially important in young people during sleep onset and light sleep, as many vertex waves and positive occipital sharp transitions may appear "spiky",

mimicking epileptic discharges. The transition to sleep tends to produce normal paroxysmal features resembling generalized epileptic discharges. Sharp transitions are detected in more than 90% of patients during the transition to sleep, which may lead to misdiagnosis as epileptic discharges. Benign epileptiform transients of sleep and rhythmic midtemporal theta bursts at sleep onset may also appear similar to epileptic discharges (6). Many types of epilepsy occur only during sleep. Interictal epileptiform discharges on the EEG are also most likely to be activated during deep NREM sleep stage N3. Normal variants, which are most likely to be misdiagnosed as epileptiform, are usually seen during sleep transition and tend to disappear in deeper sleep (4). Sleep deprivation has no effect on EEG monitoring of epilepsy in some studies (21).

Conclusion

Focal or generalized epileptic discharges were reported in 3.5% of children without epilepsy. The majority of that EEG findings disappeared by early adolescence without the development of seizures or epilepsy (6). To correctly interpret an abnormal EEG, one must first have the ability to identify normal patterns. There is no gold standard for EEG interpretation (22). There is no objective definition of interictal epileptiform discharges. Even experienced EEG experts may sometimes disagree on the diagnosis of interictal epileptiform discharge. For this reason, EEG interpretation cannot be objective due to poor inter-observer reliability (8). It is necessary to be strict in order to diagnose epilepsy on EEG (7). When interpreting EEG, if the examined waveform is similar in terms of epileptiform and non-epileptiform features, a non-epileptiform interpretation is preferred. In this case, the "2-minute rule" may be useful to avoid erroneous interpretation of "suspicious" waveforms. If a waveform is examined for more than 2 minutes and no decision can be made, it is recommended to interpret it as a benign feature of the EEG. EEG findings should always be interpreted within the clinical context of the recording (3). Interictal epileptic discharges in the temporal lobe are frequently observed in patients with temporal lobe epilepsy (1). Because of the increased yield of serial EEGs, it is recommended that three or four recordings be taken in patients with suspected epilepsy. One of the recordings should include sleep combined with sleep deprivation. After this point, the yield of serial EEGs is relatively low (4). Recording nocturnal sleep increases the likelihood of detecting seizures and interictal epileptiform discharges compared with routine daytime EEG (23). Normal patterns that occur during drowsiness and rest are the most common EEG findings that are erroneously interpreted as abnormal. In general, these benign patterns occur during drowsiness and light sleep, without interrupting background

EEG activity (16, 18). An abnormality on the EEG should be reported only if definite (18). EEG helps with clinical decision making. Diagnosis is made based on the clinic, not the EEG. The basis of EEG interpretation is pattern recognition, and reading EEG is an art rather than a science. Various attempts have been made to develop criteria to define interictal epileptiform discharges, but these criteria have not yet been standardized. Recognition of normal physiological features and the wide range of normal EEG variants, is important for correct interpretation of the EEG (24).

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Chapter 3

Chromatographic Methods for Analyzing Drug-Biological Interactions

Selen Duygu ÇEÇEN[1]

Introduction

A drug molecule typically possesses key characteristics such as optimal molecular weight (usually below 500 Da for oral drugs), balanced lipophilicity (logP between 0 and 5), hydrogen bonding capacity, solubility, and chemical stability. These properties influence how a drug interacts with proteins and membranes, ultimately affecting its efficacy, bioavailability, and safety [1, 2].

Interactions between chemical and biological molecules play a crucial role in many clinical and pharmacological processes. Drug-protein and drug-membrane interactions are fundamental to drug action and pharmacokinetics, shaping a drug's efficacy, distribution, metabolism, and potential toxicity [3, 4]. Many drugs exert their effects by binding to specific proteins, such as receptors, enzymes, or ion channels, to trigger a biological response. The strength and specificity of this binding determine a drug's potency and selectivity, while unintended interactions with off-target proteins can lead to side effects or toxicity [5]. Additionally, plasma protein binding—primarily to albumin or α1-acid glycoprotein—affects drug distribution by altering the free (active) drug concentration in circulation. Highly protein-bound drugs may have reduced bioavailability, requiring dose adjustments to maintain therapeutic effects [6]. Notably, approximately 43% of the 1500 most common drugs exhibit at least 90% serum protein binding [7].

Beyond protein interactions, a drug's ability to reach its target often depends on its ability to cross biological membranes. This makes drug-membrane interactions critical for absorption, distribution, and cellular uptake [8]. Physicochemical properties such as lipophilicity and charge determine whether a

[1] Arş. Gör.Dr. Istanbul University-Cerrahpaşa, Faculty of Pharmacy, Department of Analytical Chemistry. Orcid No: 0000-0002-3628-9892

drug passively diffuses through lipid bilayers or relies on active transport mechanisms. Hydrophilic drugs typically require carrier-mediated uptake or transporter proteins, while lipophilic drugs more easily traverse membranes [9]. However, efflux transporters like P-glycoprotein (P-gp), BCRP (breast cancer resistance protein), and MRPs (multidrug resistance proteins) actively expel certain drugs from cells, reducing their intracellular concentration and contributing to drug resistance in conditions such as cancer and bacterial infections [10-12]. In some cases, drugs can interact directly with membrane phospholipids or cholesterol, altering membrane fluidity and integrity, which may impact cellular function or contribute to cytotoxic effects [13, 14].

1. Importance of Analyzing Drug-Protein and Drug-Membrane Interactions

Understanding drug-protein and drug-membrane interactions is essential for optimizing drug design, improving therapeutic efficacy, and minimizing adverse effects. These interactions influence key pharmacokinetic and pharmacodynamic properties, including ADME and toxicity. A drug's therapeutic effect depends on its ability to bind selectively to its intended protein target while minimizing off-target interactions [15].

Plasma protein binding plays a crucial role in drug distribution by affecting the concentration of free drug available in circulation. Highly protein-bound drugs may exhibit reduced bioavailability, slower clearance, and prolonged half-lives. For example, drugs extensively bound to albumin may have altered distribution patterns, which can influence their effectiveness in specific tissues [16]. For intracellular drug targets, membrane permeability is a determining factor. Lipophilic drugs, which tend to show high retention in IAM chromatography, generally permeate membranes efficiently, whereas hydrophilic drugs often require active transport mechanisms [17]. Efflux transporters such as P-gp, BCRP, and MRPs further regulate intracellular drug accumulation by pumping molecules out of cells, impacting drug effectiveness and resistance Non-specific protein binding or membrane interactions can also contribute to toxicity. Amphiphilic drugs, for instance, may insert into lipid bilayers and disrupt membrane integrity, leading to cytotoxic effects [18].

To analyze drug-protein and drug-membrane interactions, various chromatographic techniques are widely employed. These include AC, HPLC-based plasma protein binding assays, IAM chromatography, and liposome-based methods. These techniques provide critical insights into binding affinities, permeability, and distribution patterns, aiding in drug development and optimization.

2. Pre-processing steps for chromatographic analysis of drug-protein and drug-membrane interactions

Several chromatographic techniques require specific pre-processing steps to ensure accurate analysis of drug-protein and drug-membrane interactions. Equilibrium dialysis and ultrafiltration are commonly used before HPLC-based plasma protein binding assays to separate free and bound drug fractions [19].

In equilibrium dialysis, a drug-protein mixture is placed in a semi-permeable membrane chamber, allowing only the free drug to diffuse across into a buffer solution over several hours, after which drug concentrations in both compartments are measured by HPLC or LC-MS to determine binding affinity [20]. Ultrafiltration, on the other hand, relies on centrifugal filters with molecular weight cutoffs (~30 kDa) to retain protein-bound drug while allowing free drug to pass through; after centrifugation, the filtrate is analyzed chromatographically [21]. For size-exclusion chromatography (SEC) studies of protein interactions, ultrafiltration or desalting columns help remove unbound drug molecules before injection into the column [22]. When analyzing cellular uptake and efflux using HPLC or LC-MS, pre-treatment of cell cultures is necessary. This involves exposing cells to a known drug concentration, washing them to remove extracellular drug, lysing the cells via sonication or chemical lysis buffers, centrifuging to clear debris, and collecting the supernatant for chromatographic analysis [23]. For drug-membrane interactions, IAM chromatography and liposome-based chromatography do not require additional pre-processing, as the drug is directly injected into specialized columns designed to mimic biological membranes [24, 25]. Additionally, RP-HPLC is widely used for lipophilicity measurements (logP/logD) without extra sample preparation, as it directly assesses the drug's partitioning between aqueous and organic phases [26]. However, parallel artificial membrane permeability assays (PAMPA), used to evaluate passive permeability, involve applying the drug to an artificial lipid barrier before chromatographic quantification [27]. These preparation steps are critical to ensure reliable measurement of drug interactions, helping to optimize drug design and predict pharmacokinetic behavior.

Besides the high need of the pre-processing methods for several chromatographic techniques, other may need lower steps and time for interaction analyses such as HPLC-based plasma protein binding assays and Cellular Uptake and Efflux Assays, some chromatographic techniques such as AC, IAM Chromatography, Liposome-Based Chromatography and PAMPA may need those long processes. Table 1 gives the chromatographic techniques and need for preprocessing processes.

Table 1. Chromatographic techniques and need for preprocessing processes for analyzing drug-protein interactions [15, 26, 28-30].

Chromatographic Technique	**Target**	**Pre-Processing Step Required?**	**Preparation Steps**
Affinity Chromatography	Drug-Protein	No	Directly measures drug binding to immobilized proteins (e.g., enzymes, receptors).
HPLC-Based Plasma Protein Binding Assays	Drug-Protein	Yes	Equilibrium Dialysis or Ultrafiltration to separate free and bound drug fractions before HPLC analysis.
Immobilized Artificial Membrane (IAM) Chromatography	Drug-Membrane	No	Drug is directly injected into the IAM column to assess membrane interaction.
Liposome-Based Chromatography	Drug-Membrane	No	Direct injection into a liposome-embedded column to mimic biological membranes.
Reverse-Phase HPLC (RP-HPLC) for Lipophilicity (logP/logD measurement)	Drug-Membrane	No	Direct measurement of partitioning behavior.
Size-Exclusion Chromatography (SEC) for Protein Binding	Drug-Protein	Yes	Ultrafiltration or desalting to remove unbound drug before analysis.
Cellular Uptake and Efflux Assays (HPLC/LC-MS based)	Drug-Membrane	Yes	Cell Culture Pre-Treatment, Cell Lysis, or Equilibrium Dialysis to isolate intracellular and extracellular drug fractions before HPLC/LC-MS analysis.
Parallel Artificial Membrane Permeability Assay (PAMPA)	Drug-Membrane	No	Drug is applied to an artificial membrane, and permeability is measured directly.

3. Chromatographic Systems for Analyzing Drug-Protein and Drug-Membrane interaction

Chromatographic techniques provide valuable insights into drug-protein and drug-membrane interactions, which are crucial for understanding drug behavior in biological systems in terms of medicine [31]. Although analyzing interactions between drugs and protein or membrane in terms of chromatographic systems, these techniques are possible to be classified according to their stationary and mobile phases. These methods are a type of biomimetic chromatographic methods because they need to mimic drug interactions as pre-processing or in the chromatography environment.

Non-polar reversed-phase (RP) stationary phases with aliphatic ligands, such as C-18 and C-8, are the most commonly used biomimetic phases that can mimic the non-polar interior of a biological membrane. These techniques analyze substances based on their potential interactions with blood proteins and biological membranes, which contain transport proteins and membrane receptors embedded in a phospholipid bilayer. Reversed-phase chromatography (RP) and micellar liquid chromatography (MLC) are commonly conducted using these phases [32, 33]. Other stationary phases as improved techniques can include several designed including covalently AIM or special components of cell membranes such as Cholesterol, sphingomyelin (SPH) and phosphatylethanolamine [33, 34]. Stationary phases with active cell membranes (CMSP) or phospholipid bilayers immobilized—liposomes. The examples of stationary phase designs can be improved using special fragments of cell membranes [35].

In chromatography systems that use stationary phases with immobilized membranes or membrane fragments, the interaction behavior of the analyzed drugs influences their amounts and the retention times that means a drug-protein(s) dependent diverse volume of distribution patterns. While some commercial columns with protein-immobilized stationary phases are available, special care may still be needed to optimize the system and maintain the activity of the membrane-bound proteins [33].

Affinity chromatography is widely used for studying drug-protein binding by immobilizing target proteins, such as serum albumin or enzymes, onto a solid support. A drug solution is passed through the column, allowing selective binding, while unbound molecules are washed away. This method helps determine binding affinity and specificity, making it essential in drug discovery and pharmacokinetics [29, 36, 37].

Another powerful approach is high-performance size-exclusion chromatography (HPSEC), which separates molecules based on size, distinguishing between free drugs and drug-protein complexes. Larger protein-

drug complexes elute first, followed by smaller, unbound drug molecules. HPSEC is particularly useful for analyzing monoclonal antibody-drug conjugates, protein aggregation, and the stability of drug formulations in biological fluids [38, 39]. Similarly, frontal analysis chromatography provides a quantitative assessment of drug-protein interactions by continuously introducing a drug solution into a protein-immobilized column until binding sites are saturated. Measuring the concentration of free drug in the eluent allows for determining binding constants and interaction kinetics, making this method ideal for evaluating highly bound drugs such as warfarin or diazepam [40].

Hydrophobic interactions also play a significant role in drug-protein binding, which can be studied using reversed-phase liquid chromatography (RP-LC) and hydrophobic interaction chromatography (HIC) [41]. RP-LC exploits differences in hydrophobicity by using a nonpolar stationary phase and a polar mobile phase, allowing for the differentiation of free and bound drug fractions. This technique is widely applied in pharmacokinetic studies to quantify protein binding and drug solubility under physiological conditions. In contrast, HIC uses a decreasing salt gradient to separate drug-protein complexes based on their hydrophobicity, making it particularly useful for amphiphilic drug interactions with lipid-binding proteins and evaluating drug stability [42].

Beyond protein interactions, chromatographic methods also provide insight into drug-membrane interactions, which are critical for understanding drug absorption, permeability, and bioavailability. IAM chromatography simulates biological membranes by incorporating phospholipids onto a chromatographic support. The retention time of a drug in an IAM column correlates with its membrane permeability, making this technique valuable for predicting passive diffusion, blood-brain barrier penetration, and oral bioavailability. A similar approach, liposome chromatography, utilizes phospholipid-coated columns to mimic the lipid bilayer environment, helping assess drug retention, membrane affinity, and cytotoxicity of lipophilic compounds.

Other biomimetic techniques, such as MLC and biomimetic chromatography, further refine the study of drug-membrane interactions. MLC uses surfactant micelles in the mobile phase to create a pseudo-stationary phase that mimics biological membranes, allowing for the analysis of hydrophobic drug-lipid interactions and optimization of lipid-based drug formulations. Biomimetic chromatography, on the other hand, employs synthetic membrane-like materials to replicate phospholipid bilayers, enabling the assessment of drug permeability and transport across biological barriers such as the gastrointestinal tract and the blood-brain barrier [43].

Additionally, chiral chromatography plays a crucial role in evaluating drug interactions by separating enantiomers to study their stereoselective binding to proteins or membranes. Since enantiomers can exhibit distinct pharmacokinetics and pharmacodynamics, this technique ensures that potential differences in metabolism, bioavailability, and toxicity are identified during drug development [44, 45]. By combining these chromatographic approaches, researchers can gain a comprehensive understanding of how drugs interact with biological targets, guiding the development of safer and more effective pharmaceutical compounds.

Chromatographic techniques play a crucial role in studying drug interactions with both proteins and membranes, offering diverse applications based on the nature of the drug, the interaction type, and analytical requirements. Reverse-phase HPLC is widely used for both drug-protein and drug-membrane studies due to its ability to assess hydrophobic interactions and quantify drug partitioning behavior. It is particularly valuable for small-molecule drug analysis, providing high sensitivity and moderate infrastructure requirements [46]. AC, commonly employed in drug-protein studies, enables highly specific binding assessments using immobilized target proteins, while its variation, immobilized artificial membrane (IAM) chromatography, is tailored for drug-membrane interactions by incorporating phospholipid-coated columns to simulate permeability studies [46]. Size-exclusion chromatography, though primarily used in protein research to study complex formation and aggregation, has limited application in membrane studies due to its reliance on molecular size rather than direct membrane interaction analysis [28]. Liposome chromatography, on the other hand, is designed for membrane research, utilizing lipid-incorporated stationary phases to evaluate drug accumulation and distribution in membrane-like environments [47]. Hydrophobic interaction chromatography is applicable to both drug-protein and drug-membrane studies by exploiting hydrophobicity differences, making it useful for protein-ligand binding assessments as well as interactions with lipid bilayers [48]. Ion-exchange chromatography is particularly beneficial for analyzing charge-based interactions, whether in the context of protein binding or membrane interactions involving charged phospholipids and ion channels [49].

The complexity of these methods varies, with RP-HPLC and IAM chromatography offering moderate ease of use, while AC, liposome chromatography, and ion-exchange chromatography demand more specialized setups. Sensitivity also differs across techniques, with AC and IAM chromatography, providing the highest specificity for detecting weak or transient interactions, while RP-HPLC excels in quantitative analysis. Ultimately, the selection of a chromatographic technique depends on the drug's physicochemical properties, the nature of its interaction with proteins or membranes, and

laboratory infrastructure, with RP-HPLC being a versatile choice for general hydrophobicity assessments, affinity and IAM chromatography excelling in specificity-driven studies, and ion-exchange and liposome chromatography offering valuable insights into charge-dependent and membrane-penetration properties (Table 2) [5, 36, 37, 50-52].

4. Studies for drug-protein/membrane interactions in the literature

The interaction between drugs and biological macromolecules such as proteins and membranes play a crucial role in understanding their pharmacokinetics and pharmacodynamics. Chromatographic techniques have been widely used to investigate these interactions, providing valuable insights into drug binding and distribution.

Affinity chromatography has been widely used to investigate drug-protein interactions. For example, aspirin's binding to albumin, a major plasma protein, has been demonstrated using AC coupled with HPLC and UV detection [53]. Rifampicin, an anti-tuberculosis drug, was also studied for its interaction with cytochrome P450 using the same technique [54].

Table 2. Comparison of chromatographic techniques commonly used for drug interaction analysis [29, 30, 36, 37, 50, 52].

Technique	**Interactions**	**Usage Frequency**	**Physical Properties of Drugs Analyzed**	**Application Complexity**	**Infrastructure Needs**	**Strengths**	**Limitations**
Affinity Chromatography	Drug-Protein	High	Binding affinity, specificity, protein-drug complex	Medium	Moderate (requires protein ligands)	High specificity, detailed interaction analysis	Time-consuming, expensive, requires purified proteins
HPLC-Based Plasma Protein Binding Assays	Drug-Protein	High	Plasma protein binding, free drug concentration	Low	Low (standard HPLC setup)	Simple, fast, standard method	Does not provide information on binding sites or interaction types

Size-Exclusion Chromatography (SEC)	Drug-Protein	Medium	Drug-protein complexes (size-based separation)	Medium	Moderate (HPLC system with SEC column)	Separates based on size, useful for complex analysis	Limited to large drug-protein complexes, can be less sensitive for small molecules
Hydrophobic Interaction Chromatography (HIC)	Drug-Protein	Medium	Hydrophobic interactions, drug-protein binding	High	Moderate (HPLC system)	Identifies hydrophobic binding sites, good for structural studies	Requires optimization of buffers, can be complex
Ion-Exchange Chromatography (IEX)	Drug-Protein	Low	Electrostatic interactions, charge-based separation	Medium	Moderate (HPLC system)	Useful for charged molecules, good for protein characterization	Less effective for neutral drugs, can be less specific
Immobilized Artificial Membrane (IAM) Chromatography	Drug-Membrane	High	Lipophilicity, permeability, drug-membrane interactions	High	High (requires IAM columns and HPLC system)	Mimics biological membranes, good for permeability studies	Expensive, requires specialized columns and setup
Liposome-Based Chromatography	Drug-Membrane	Medium	Membrane binding, lipid bilayer interaction, solubility	High	High (liposome preparation and HPLC system)	Good for studying membrane disruption and cytotoxicity	Complex preparation, can be expensive
Biomimetic Phospholipid Chromatography	Drug-Membrane	Medium	Lipid-drug interaction, membrane components	High	High (HPLC system with phospholipid-based column)	Reflects natural membrane properties, useful for drug interactions	Complex and expensive setup, limited availability of specific columns
Reverse-Phase HPLC (RP-HPLC)	Drug-Membrane	High	Lipophilicity, solubility, permeability (logP/logD)	Medium	Moderate (standard HPLC system)	Quick, widely used, easy to interpret for lipophilicity	Limited in providing direct membrane interaction data

Similarly, promethazine, an antihistamine, was analyzed for its binding to serum albumin through affinity chromatography [6]. Additionally, clopidogrel, a cardiovascular drug, was assessed for its interaction with P-glycoprotein using LC-MS/MS-based affinity chromatography [55].

HPLC-based plasma protein binding assays have provided additional insights into drug-protein interactions. For instance, ibuprofen, an anti-inflammatory drug, was studied for its binding to α1-acid glycoprotein [56]. Similarly, metformin, an antidiabetic drug, was analyzed for its interaction with the same protein using HPLC-UV detection.

Chromatographic techniques have also been employed to explore membrane receptor interactions. Tamoxifen, an anti-breast cancer drug, was studied using cell membrane chromatography coupled with HPLC and liposome preparations to assess its receptor interactions [57]. Doxorubicin, another anticancer drug, was analyzed using RP-HPLC for its affinity toward membrane receptors [58]. In addition, a combination of antifungal agents, including tebuconazole, triticonazole, and hexaconazole, was examined for their interaction with membrane transporters using RP-HPLC with MS/MS detection [59].

Several studies have focused on drug-membrane interactions using biomimetic phospholipid membrane chromatography. Paclitaxel, an anticancer drug, was evaluated for its membrane affinity using phospholipid monolayer chromatography [60]. Hydroxychloroquine, a drug for autoimmune diseases, was examined through similar methods [61]. Furthermore, liposome-based chromatography has been applied to study drug-membrane interactions, such as in the case of diclofenac, where its affinity for phospholipids was investigated [62]. Cisplatin, a widely used chemotherapeutic agent, was also assessed for its interaction with phospholipids using liposome chromatography [63].

These studies highlight the critical role of chromatographic methods in evaluating drug interactions with biological targets. The diverse techniques employed, such as affinity chromatography, biomimetic membrane chromatography, and RP-HPLC, provide a comprehensive understanding of drug behavior in biological systems (Table 3). Future research should continue exploring these methodologies to enhance drug development and therapeutic efficacy.

Conclusion

Chromatographic techniques enable a robust and versatile approach to examine drug-protein and drug-membrane interactions, both of which are essential for understanding drug behavior in human biological systems. Providing accurate and reproducible techniques to analyze affinity, kinetics of interaction, permeability and

partition characteristics, they contribute considerably to drug discovery, development and formulation. While protein-based chromatographic techniques simulate biological barriers to predict drug transport and bioavailability, membrane mimic approaches facilitate the estimation of drug binding to plasma proteins and receptors. Their integration enables extensive assessment of drug interactions under physiologically relevant conditions, improving the precision of the pharmacokinetic predictions and optimizing therapeutic outcomes. As chromatographic technologies continue to evolve, their application in pharmaceutical research will continue to be essential to advance drug development and ensure the safety and efficacy of new therapeutic agents

Table 3. Several drug interactions studies in the literature.

No	Tested Drugs	Tested Proteins	Type (Drug-Protein / Drug-Membrane)	Technique Used	Equipment Used	Disease Targeted by Drug	Reference
1	Aspirin	Albumin	Drug-Protein	Affinity Chromatography	HPLC, UV Detector	Cardiovascular diseases	[53]
2	Paclitaxel	Phospholipid Bilayer	Drug-Membrane	Biomimetic Phospholipid Membrane Chromatography	HPLC, Phospholipid Monolayer	Cancer (e.g., Breast Cancer)	[60]
3	Ibuprofen	α1-Acid Glycoprotein	Drug-Protein	HPLC-Based Plasma Protein Binding Assays	HPLC, UV/Fluorescence Detector	Inflammatory Diseases	[56]
4	Tamoxifen	Membrane Receptors	Drug-Membrane	Cell Membrane Chromatography	HPLC, Liposome Preparations	Breast Cancer	[57]
5	Diclofenac	Phospholipids	Drug-Membrane	Liposome-Based Chromatography	HPLC, Liposome Preparations	Pain Management (e.g., Osteoarthritis)	[62]
6	Rifampicin	Cytochrome P450	Drug-Protein	Affinity Chromatography	HPLC, UV Detector	Tuberculosis	[54]
7	Doxorubicin	Membrane Receptors	Drug-Membrane	RP-HPLC	HPLC, UV Detector	Cancer (e.g., Leukemia)	[58]
8	Cisplatin	Phospholipids	Drug-Membrane	Liposome-Based Chromatography	HPLC, Liposome Preparations	Cancer (e.g., Ovarian Cancer)	[63]
9	Metformin	α1-Acid Glycoprotein	Drug-Protein	HPLC	HPLC, UV Detector	Type 2 Diabetes	[64]
10	Hydroxychloroquine	Phospholipids	Drug-Membrane	Biomimetic Phospholipid Membrane Chromatography	HPLC, Phospholipid Monolayer	Autoimmune Diseases (e.g., Lupus)	[61]
11	Tebuconazole, Triticonazole, Hexaconazole, Penconazole, Uniconazole	Membrane Transporters	Drug-Membrane	RP-HPLC	HPLC, MS/MS Detector	Fungal Infections	[59]
12	Promethazine	Serum Albumin	Drug-Protein	Affinity Chromatography	HPLC, UV Detector	antihistaminic	[6]
13	Clopidogrel	P-glycoprotein	Drug-Protein	Affinity Chromatography	LC, MS/MS Detector	Cardiovascular Diseases	[55]

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