MATRIX INFLUENCE IN DETERMINING ANTIPARASITIC BY HPLC

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UTICAJ MATRIKSA NA ODREĐIVANJE ANTIPARAZITIKA HPLC METODOM

Apstrakt

Tečna hromatografija visokih performansi (HPLC, tečna hromatografija pod visokim pritiskom) je oblik kolonske *hromatografije* koji se često koristi u *analitičkoj hemiji*. HPLC se koristi za razdvajanje komponenti iz smese na osnovu hemijskih interakcija između supstance koja se analizira i stacionarne faze u koloni. Princip rada HPLC-a je forsiranje prolaska analizirane supstance (ili *smeše*) kroz kolonu (cev napunjenu materijalom sitnih čestica, a time i velike površine) pumpanjem tečnosti (mobilna faza) pod visokim pritiskom kroz kolonu. Unosi se mala zapremina uzorka u tok mobilne faze i na osnovu specifičnih hemijskih i fizičkih interakcija, dolazi do različitog zadržavanja komponenata smeše. Vreme zadržavanja zavisi od prirode supstance koja se analizira, stacionarne faze i sastava mobilne faze. Vreme za koje se supstanca eluira (dođe do kraja kolone) naziva se retenciono vreme i one je karakteristično za određenu supstancu. Korištenje visokog pritiska povećava linearnu brzinu i daje komponentama manje vremena za zadržavanje, što poboljšava rezoluciju hromatograma. Koriste se uobičajeni *rastvarači*, čisti ili u bilo kojoj kombinaciji (npr. voda, *metanol*, organski rastvarači, itd). Voda može sadržavati i neki *pufer*, kako bi se poboljšalo razdvajanje. Moguće je koristiti i gradijentno eluiranje, što podrazumeva promenu sastava mobilne faze u toku eluiranja. Uređaj za HPLC se sastoji od sledećih komponenata: rezervoar mobilne faze, pumpe i injektora. Detektor ima važnu ulogu da određivanja komponenti koje izlaze iz kolone nakon eluiranja. Detektor generiše električni signal koji je proporcionalan intenzitetu neke osobine mobilne faze ili supstance koja se eluira. Tipovi detektora u HPLC-u su: UV-VIS detektor, fluorescentni detektor, elektrohemijski detektor, detektor indeksa loma i maseni spektrometar (MS). Uticaj matrixa uzorka koji se analizra ima veliki uticaj na izlazne analiticke rezultate. Kako

bi se eliminisao uticaj matrixa pozeljno je uraditi kalibraciju kroz matrix, na blank uzorku koji ne sadrzi analite od interesa (Olives, 2006). U našoj studiji smo ukazali na značaj kalibracije kroz matrix, kako bismo neutralisali negov nepovoljan uticaj na rezultat. S tim ciljem je urađena i analiza uzoraka mesa ribe koja je spajkovana sa razlicitim koncentracijama parazitika. Uzorci su ekstrahovani sa acetonitrilom u prisustvu soli, anhidrovanog magnezijum sulfata i natrijum acetate. Kvantifikacija prečišenog ekstrakta je urađena na Thermo HPLC-u, sa UV detektorom. Na osnovu sprovedenog ispitivanja utvrđeno je da procenat recovery kod kalibracije kroz matrix se krece u opsegu od 75-95 % a u slučaju kalibracije sa standardima kreće se u opsegu od 40-55%. Zbog značaja praćenja parazitika u mesu ribe neophodno je optimizovati uslove analize s ciljem dobijanja pouzdanih rezultata i pračenja kvaliteta proizvoda.

Ključne reči: HPLC, parazitici, kalibracija, matriks
Keywords: HPLC, antiparasitics, calibration, matrix

INTRODUCTION

High performance liquid chromatography is basically a highly improved form of column chromatography. Instead of a solvent being allowed to drip through a column under gravity, it is forced through under high pressures of up to 400 atmospheres. That makes it much faster. It also allows the use of much smaller particle size for the column packing material which gives a much greater surface area for interactions between the stationary phase and the molecules flowing past it. This allows a much better separation of the components of the mixture. The other major improvement over column chromatography concerns the detection methods which can be used. These methods are highly automated and extremely sensitive. **Normal phase HPLC** -This is essentially just the same as in thin layer chromatography or column chromatography. Although it is described as „normal", it isn't the most commonly used form of HPLC. The column is filled with tiny silica particles, and the solvent is non-polar - hexane, for example. A typical column has an internal diameter of 4.6 mm (and may be less than that), and a length of 150 to 250 mm. Polar compounds in the mixture being passed through the column will stick longer to the polar silica than non-polar compounds will. The non-polar ones will therefore pass more quickly through the column. **Reversed phase HPLC** - In this case, the column size is the same, but the silica is modified to make it non-polar by attaching long hydrocarbon chains to its surface - typically with either 8 or 18 carbon atoms in them. A polar solvent is used - for example, a mixture of water and an alcohol such as methanol. In this case, there will be a strong attraction between the polar solvent and polar molecules in the mixture being passed through the column. There won't be as much attraction between the hydrocarbon chains attached to the silica (the stationary phase) and the polar molecules in the solution. Polar molecules in the mixture will therefore spend most of their time moving with the solvent. Non-polar compounds in the mixture will tend to form attractions with the hydrocarbon groups because of van der Waals dispersion forces. They will also be less soluble in the solvent because of the need to break hydrogen bonds as they squeeze in between the water or methanol molecules, for example. They therefore spend less time in solution in the solvent and this will slow them down on their way through the column. That means that now it is the polar molecules

that will travel through the column more quickly. Reversed phase HPLC is the most commonly used form of HPLC. There are several ways of detecting when a substance has passed through the column. A common method which is easy to explain uses ultra-violet absorption. Many organic compounds absorb UV light of various wavelengths. If there is a beam of UV light shining through the stream of liquid coming out of the column, and a UV detector on the opposite side of the stream, a direct reading of how much of the light is absorbed can be obtained. The amount of light absorbed will depend on the amount of a particular compound that is passing through the beam at the time different compounds absorb most strongly in different parts of the UV spectrum. Methanol, for example, absorbs at wavelengths below 205 nm, and water below 190 nm. If methanol-water mixture is used as the solvent, therefore a wavelength greater than 205 nm to avoid false readings from the solvent should be used. The sample matrix can have a profound impact on the analytical results for an HPLC method. As a result of this, a matrix-based calibration curve is recommended almost universally. There are several goals of sample preparation. One is to remove materials that might interfere chromatographically with the analyte, another is to get acceptable recovery of the analyte and a third is to remove "column killers" – those matrix component that shorten column lifetime. A common way to test recovery is to spike known amounts of reference standard into a blank matrix, or placebo, mix it well, then perform sample extraction. The recovered amount then can be compared to the amount spiked into the matrix to determine recovery. This is done by comparing the signal of the extracted sample to the signal obtained from pure reference standard. Although it is ideal to get 100% recovery of the analyte, this seldom occurs. In many cases, recovery of 80-95% can be obtained, but in others, recovery may be below 50%. Although the method usually will perform more reliably and reproducibly the higher the recovery, this is not necessarily the case or a requirement for a successful method 86% of the sample that one spikes into a blank matrix, it is expected that one will also recover 86% of the analyte from a real sample. Then the extracted samples can be directly related to the true amount of analyte present. The simplest way to do this is to use a matrix-based calibration curve. This is done by spiking different concentrations of reference standard into blank matrix, extracting the standard back out, and using the resulting solutions to construct the calibration curve. This will correct for the low recovery from the sample matrix of both real samples and the calibrators so that the reported values should be close to the real values. Because the matrix can have such a profound influence on the final results, it is recommended to match the matrix for the calibration curve to the sample. Sometimes one can get away without using a matrix-based calibration curve, but this should be done only when it is shown that there is no difference in the analytical results with and without the matrix present.

MATERIAL AND METHODS

In the present study used the flesh of fresh fish that is spiked with a known concentration of antiparasitic. All standards are satisfactorily high HPLC purity. Niclosamide (2',5- dichloro-4-nitrosalicylanilide) were obtained from Sigma (St. Louis, MO, USA; USP or BP grade; purity above 98%). HPLC methanol and acetonitrile were used. The HPLC system was Thermo. The flow-rate employed was 1 ml min -t. For determination was used UV detector at 254 nm.

Standard solutions of the antiparasitics niclosamide 0.0023g were dissolved in acetonitrile to obtain concentration of 100 mg/mL. A volume of 1 mL of solution was diluted to 10 mL with acetonitrile to get concentration of 10 mg/mL.

RESULTS AND DISCUSSION

We analyzed 15 samples spiked fish meat with different concentration of niclosamide and we found that recovery goes from 75 to 94% when we calibrate through matrix, and when not about 45%. In the study of HPLC determination of niclosamide Schreier et al. (1996) found that recovery of NIC from rainbow trout (n = 7) fortified at 0.04 mu g/g was 77 +/- 6.5% and from channel catfish (n = 7) fortified at 0.02 mu g/g was 113 +/- 11%. NIC detection limit was 0.0107 mu g/g for rainbow trout and 0.0063 mu g/g for catfish. Percent recovery of incurred radioactive residues by this method from catfish exposed to [C-14] NIC was 89.3 +/- 4.1%. Percent recoveries of NIC from fortified storage stability tissue samples for rainbow trout (n = 3) analyzed at 5 and 7.5 month periods were 78 +/- 5.1 and 68 +/- 2.4%, respectively. Percent recoveries of NIC from fortified storage stability tissue samples for channel catfish (n = 3) analyzed at 5 and 7.5 month periods were 88 +/- 13 and 76 +/- 21%, respectively .The lowest recovery after ten replicate injections of known samples was 100.2% and the highest 104.2%, with an overall mean deviation from 100% of 1.1% (Elsa, 1997). The antiparasitic are drugs that have been used for about 50 years in crop protection, human and animal health. The treatment of antiparasitic diseases is a topic of great importance in these days for the pharmaceutical industry, because it becomes increasingly necessary to improve the pharmaceutical formulations due to the mix presentation of these diseases in many animals (César Soto, 2013).

CONCLUSION

The use of the HPLC method allows a selective and quantitatively accurate analysis of several anthelmintics in veterinary dosage forms. The chromatographic method is sufficiently specific, precise and sensitive for the purpose of analytical characterisation of niclosamide. Preliminary results not given here also showed that the method was applicable for the analysis of other anthelmintic.

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