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FLUOREMETRIC DETERMINATION OF AMPICILLIN IN MILK WITH PRECOLUMN DERIVATIZATION BY HIGH PERFORMANCE LIQUID CHROMATOGRAPHY

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Yüksek Performanslı Sıvı Kromatografisi ile Sütte Ampisilinin Kolon Öncesi Derivatizasyonla Fluorometrik Tayini

Summary: Fluoremetric determination of ampicillin in milk with precolumn derivitization has been achieved . Separation of fluorometric ampicillin has been accomplished by means of High Performance Liquid Chromatography (HPLC) using C18 column. Mobile phase has chosen as $HClO_4$+ Methanol (55:45, v/v) which gave good separation. Detection was made at excitation as 346 nm and emissions as 422 nm. Standard ampicillin and milk samples added with ampicillin have been derivatized with 0.4 M citric acid solution that dissolved in 7% formaldehyde solution. Simple extraction step without further clean up has been accomplished with milk sample. No other interferences observed. Fluoregenic properties of derivatize ampicillin allowed to measure as low as 10 ng /ml that is much better than UV detection limit. Recovery of ampicillin from ampicillin added milk has been found as 98.5-112%. This method is simple and much more sensitive to measure ampicillin residue in milk sample.

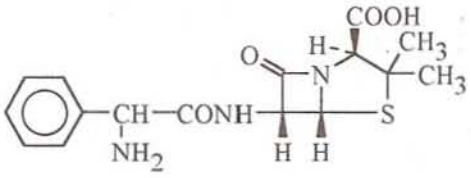
Key Words: Ampicillin, Derivatization, Milk, HPLC, Fluoresence

Özet: Kolon öncesi derivatizasyonla sütte ampisilinin fluorometrik tayini yapıldı. Fluorometric ampisilinin separasyonu, C18 kolonu kullanılarak yüksek performanslı sıvı kromatografisi yardımıyle gerçekleştirildi. Mobil faz olarak iyi bir separasyon veren $HClO_4$ + Metanol (55:45, v/v) seçildi. Deteksiyonlar eksitasyon 346 nm ve emisyon 422 nm de yapıldı. Standart ampisilin ve ampisilin eklenmiş süt numuneleri %7 formaldehid'de çözündürülen 0.4 M sitrik asit solüsyonuyla derivatize edildi. Basit ekstraksiyon basamağı daha ileri temizlik gerektirmeden süt numunesinde başarıldı. Başka kirlilikler gözlenmedi. Derivatize ampisilinin fluorejenik özelliği UV detektör limitlerinden daha iyi olarak, en az 10 ng/ml yi ölçmeye izin verdi.Ampisilin ilave edilen sütten ampisilinin geri alınma oranı % 98.5-112 bulundu. Bu metod basit ve süt numunelerinde ampisilin kalıntısını ölçmek için çok daha duyarlıdır.

Anahtar Kelimeler: Ampisilin, Derivatizasyon, Süt, HPLC, Fluoresans

Introduction

Ampicillin is a well-known antibiotic within β-lactams group . Ampicillin has been widely used in veterinary practice recently. Ampicillin ($C_{16}H_{19}N_3O_4S$) has a molecular weight of 349.42. Ampicillin obtained from 6-Aminopenisilanic acid which has more stability and antibacterial activity then penicillin G. It is not resistance for penicillinase enzyme produced by gram +and-bacteria (1).



Ampicillin

Allowable residue content for meat and milk has been reported to be about 0.01 µg/ml (2, 3). Determination of penicillin group in milk has been reported. Some assay used for detection of β- lactam antibiotics has been found to give false results (4). Most of the method used for determination of penicillin by HPLC has a poor detection problem (5, 6). This mainly comes from utilization of ultraviolet detector. UV detection has been usually used within the range of 220-230 nm. The lowest amount can be detected through UV detector has been reported in micro (µ) quantities (5, 6, 7, 8). However, recent study with derivitization of ampicillin allows utilization of fluorescence detector to measure up to 2 ng/ml (9).

In this study, we have adapted chromatographic conditions of prior study (9) reported on sera. This study reports fluoremetric detection of ampicillin in milk with precolumn derivitization by HPLC.

Material and Methods

Materials: Ampicillin standards were prepared by dissolving 10 mg ampicillin in 100 ml dd. water. Dilution was made to reach 10-100 ng/ml. UHT

pasteurized (TS/1192) commercial milks were mixed with ampicillin to reach 10-100 ng/ml .Precolumn derivitization procedure has been made on both standard and milk added ampicillin.

Precolumn derivitization:

Derivatization agent: 7% formaldehyde (Rieadel deHaen) prepared from 35-37% stock by water dilution. 400 mM citric acid (E. Merck) solution was made by dissolving powder with formaldehyde solution (6).

500µl sample (or standard) , 100 µl 60% $HClO_4$ (E. Merck) mixed with 1000 µl citric acid solution . Mixture was left in 90 ^{o}C water bath for 2 hours. This has completed derivitization. Samples were centrifuged at 4000 rpm for 15 minute. Samples were filtered through 0.45 µm cellulose membrane. 20 µl from each sample was injected on C_8 column

Mobile phase: The mobile phase was 20 mM $HClO_4$ + Methanol (55:45 v/v) (E. Merck) and pH adjusted to 1.7 with a glass electrode. The mobile phase was filtered (Millipore, 0.45 µm) and degassed under vacuum prior use.

Chromatography: A Model LC-10AD HPLC pump (Shimadzu, Japan) was used to deliver the mobile phase isocratically at a flow rate of 1 ml/min. Samples were injected through Rheodyne 7124 injection valve (fitted with 20-ml loop). RF-10A model fluorescence detector (Shimadzu, Japan) was operated at 346 nm excitation and 422 nm emission. Samples were separated by C8 colon (150x4.6 mm, Shimadzu,Japan) at ambient temperature. Results were calculated by C-R6A model Chromatopac integrator (Shimadzu).

Results and Discussion

Standard and milk samples were derivatized prior injecting into column. This method was found very simple at the extraction step because no need further clean up samples. Besides there was no interfering natural substance in milk to yield fluorescence other compounds. Standard and milk samples gave clear florescence ampicillin peaks (see figure 1 and 2). Retention time for ampicillin was found as 3.4 minutes. Replication of method has been accomplished to verify the method.

Separation of ampicillin on C18 column material has been widely reported (5,6,7, 8). In this paper, C8 column has been also found to be convenient to separate ampicillin. Reported mobile phase for the separation of ampicillin generally consists of Acetonitrile (5, 6, 7, 9). However in this study, utilization of methanol as mobile phase could be considered more economic over acetonitrile.

Derivatization of the lowest 10 ng ampicillin/ml has been achieved and measured accurately (Table). Comparison of mean values of standard and milk ampicillin by t-test (10) indicated that there are no differences in dervatization of ampicillin mean values between milk and standard (see table).The sensitivity of instruments could be increased and standard values could be decreased to test the success of derivitization lower limit. Prior derivatization study of ampicillin in serum indicated that as low as 2 ng/ml ampicillin can be measured (9) . Recent ampicillin studies (5, 6) with UV detector indicated that ampicillin doses can be measured as low as micro (µ) quantities. This method can easily measure ng/ml quantities that are lower dose than allowable ampicillin in meat and milk (0.01 µg/ml). Recoveries of derivatize ampicillin standards were given in table. Recovery of 100 ng/ml ampicillin standard after derivitization was 102.6 %. However 10 ng ampicillin has been over estimated with recovery of 320%. This would be due to dilution mistake. Derivatize ampicillin from milk has been recovered at 98.5-112% rate.

Considering low detection limits of UV detector (5,6,7,8) and possibility of false-positive results with other methods (4). derivitization of ampicillin in milk has enhanced the detection limits and allowed rapid measurements of ampicillin in milk by HPLC.

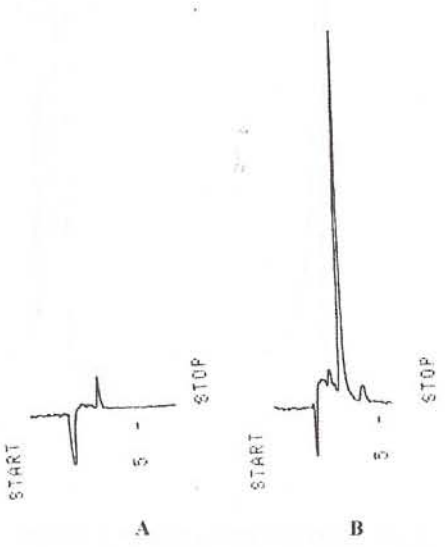


Figure 1: Full chromatograms of derivatize Ampicillin standards. (A) 10 ng ampicillin /ml (B) 100 ng ampicillin. Retention time is 3.3 minute. Separations of derivatize ampicillin have been achieved on C8 column. Fluorescence readings were set excitation at 346 nm and emission at 422 nm. Mobile phase was 20 mM $HClO_4$ (pH 1.7)-Methanol (55:45, v/v) . Flow rate was 1 ml/min.

Table. Mean and relative recovery values of derivatize ampicillin from standard and standard added milk.

Dilution	n	Mean ± SE*	Relative recovery %
Standard 100 ng ampicillin/ml	5	102.6± 5.9 [a]	102.6
Standard 10 ng ampicillin/ml	4	32.34 ± 4.9[b]	320
Milk + 100 ng ampicillin/ml	5	101.1 ±22.5[a]	98.5
Milk + 10 ng ampicillin/ml	4	32.64 ±4.5[b]	112.4

*a.b the same letters indicate no significant differences among mean values at 0.005 level.

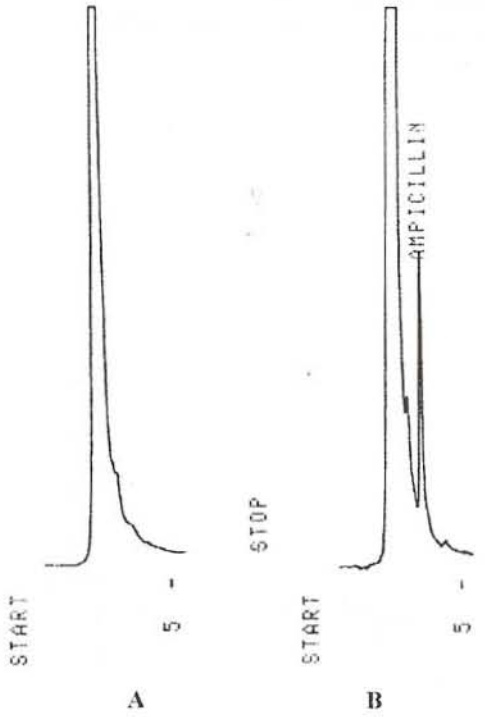


Figure 2: (A) Full chromatograms of derivatize milk prior Ampicillin standard addition. (B) Full chromatograms of derivatize milk after Ampicillin standard addition. Retention time is 3.3 minute. Separations of derivatize ampicillin have been achieved on C8 column. Fluorescence readings were set excitation at 346 nm and emission at 422 nm. Mobile phase was 20 mM $HClO_4$ (pH 1.7)-Methanol (55:45, v/v) . Flow rate was 1 ml/min.

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