

以直接酵素連結免疫吸附分析試劑測定 豬體中磺胺二甲嘧啶

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摘 要

磺胺二甲嘧啶(sulfamethazine)被懷疑具有致癌性,在動物飼料添加劑使用非常廣泛。為減少豬肉磺胺二甲嘧啶高殘留率,必須實施全國屠宰場的監視作業。傳統的檢驗方法耗時費事,因而有磺胺二甲嘧啶直接酵素連結免疫吸附劑分析法產生以作快速篩選檢測。本研究即在評估市售美國國際診斷系統公司生產之磺胺二甲嘧啶直接酵素連結免疫吸附分析試劑套組(sulfamethazine one-step enzyme-linked immunosorbent assay test kit)之性能,其結果可做為使用此試劑者之參考。包括樣品前處理,該試劑所需檢測時間約30分鐘,在平板振盪器上一次可同時分析96個樣品,其偵測極度(detection limit)為0.001 ppm。於空白的血清樣品添加0.04、0.2和0.42 $\mu\text{g/ml}$,經六重複試驗結果,平均回收率為99.8%(變異係數2.92%)。以11種常用磺胺劑:sulfamethazine, sulfamerazine, sulfadimethoxine, sulfamonomethoxine, sulfathiazole, sulfamethoxypyridazine, sulfaquinoxaline, sulfamethoxazole, sulfapyridine, sulfisoxazole 和 sulfadiazine各以四種不同濃度(包括0.001、0.01、0.1和1.0 $\mu\text{g/ml}$),進行試劑套組中之交叉反應性測試。若設定50%為抑制呈色反應干擾成立值,則上述11種磺胺劑濃度在1.0 $\mu\text{g/ml}$ 時僅有sulfamerazine, sulfisoxazole和sulfapyridine等三種磺胺劑有干擾現象。若以sulfamethazine之相對反應性為100%計算,則sulfamerazine為10.0%, sulfisoxazole為3.4%, sulfapyridine為2.0%。當上述三種磺胺劑濃度降低至0.1 $\mu\text{g/ml}$ 時則不受交叉反應性之影響。此試劑組操作簡便、敏感度高、檢測時間短、安全無污染且檢測成本日漸低廉,故可供作為豬肉中磺胺二甲嘧啶殘留之快速定性及半定量篩檢用。

前 言

豬肉及其內臟為我國主要之動物性食物,不但供給國人大部分的蛋白質來源,並且賺進不少外匯,對台灣農村經濟的繁榮助益良多。本省外銷豬肉輸出金額最近六年經統計為民國77年653,723仟美元,78年527,353仟美元,79年689,770美元,80年1,024,039仟美元,81年1,027,382仟美元,82年1,097,801仟美元⁽¹⁾。而此種經濟動物在飼養過程中常使用磺胺二甲嘧啶(sulfamethazine, SMT)、磺胺一甲氧嘧啶(sulfamonomethoxine, SMM)和磺胺二

甲氧嘧啶(sulfadimethoxine, SDM)等磺胺劑(sulfonamides)來預防或治療疾病;或添加於飼料中以維持罹患豬萎縮性鼻黏膜炎豬隻之正常增重;或與抗生素(antibiotics)、歐來金得(olaquinox)生長刺激劑合用;以增加飼料利用率達到增產目的。但是在使用上有停藥期之管制。其中磺胺二甲嘧啶被懷疑對人類具有致癌性⁽²⁾,並已確知對老鼠引起癌瘤⁽³⁾。本省冷凍及冷藏豬肉大部分外銷日本,日本亦規定外銷該國之豬肉中三種磺胺劑之殘留不得超過其公告檢驗方法之最低檢測量,即SMT 0.05 ppm, SMM 0.01 ppm, SDM 0.04 ppm⁽⁴⁾。我國衛生署規定磺胺劑殘留不得超過0.1 ppm⁽⁵⁾。近幾年

來我國冷凍及冷藏豬肉外銷日本,常有被檢出SMT殘留超過規定而遭日本退貨拒收,而我國政府主管檢驗機關—經濟部商品檢驗局亦同步將該生產廠場處以停止兩個月的分等優惠出口檢驗之處分,甚至暫時停止“優良輸日冷凍(藏)豬肉工廠推薦”,對外銷業者造成衝擊,致使外銷豬肉屠宰廠紛紛採用快速而敏感的酵素連結免疫吸附劑分析法(enzyme-linked immunosorbent assay, ELISA)作為毛豬屠宰前SMT殘留與否之篩選方法。在短短的一小時內可將進廠的原料活毛豬篩選完成,將殘留SMT超過規定者拒收退貨,無殘留者接收進廠屠宰。我國農委會自民國78年起輔導本省各家畜肉品市場設定監控站亦全部採用ELISA方法作SMT殘留篩選,並出具無SMT殘留證明書給採購工廠,以保證無磺胺二甲嘧啶之殘留。前(1992)年底日方已承認毛豬之屠前使用ELISA作SMT之篩選檢測法具有滿意管制效果。

傳統用來測定豬肉中殘留磺胺劑之方法包括比色法(colorimetric method)⁽⁶⁻⁷⁾,薄層層析篩選法(thin layer chromatographic screening method, TLC)⁽⁸⁻¹⁵⁾,高效薄層層析法(high-performance thin layer chromatographic method, HPTLC)⁽¹⁶⁻¹⁷⁾,氣相層析法(gas chromatographic method, GC)⁽¹⁸⁻²⁰⁾,氣相層析—質譜法(gas chromatographic-mass spectrometric method, GC-MS)⁽²¹⁻²⁷⁾,和高效液相層析法(high-performance liquid chromatographic method, HPLC)⁽²⁸⁻³⁵⁾。這些方法皆使用傳統之淨化程序,即使用液—液分配(liquid-liquid partition)及蒸發程序(evaporation procedure),因需作繁複的樣品萃取和淨化,而無法立即應用到大量樣品數目之經常性藥物篩選工作。而利用固相吸附作用淨化,不涉及液—液分配或蒸發大量溶劑之耗時步驟,每日工作八小時每一技術人員能處理40個樣品以上之HPLC法⁽³⁶⁾亦無法應用於毛豬屠宰前之篩選作業。

使用競爭性酵素免疫分析法(enzyme immunoassay, EIA)即ELISA作為豬血清SMT之經常性篩選作業不需作任何樣品萃取製備步驟。本方法之分析原理為以樣品中磺胺二甲嘧啶與試劑中磺胺二甲嘧啶酵素結合體(sulfamethazine enzyme conjugate)直接競爭吸附於微量測試中之對磺胺二甲嘧啶高度親合性抗體,若樣品中磺胺二甲嘧啶越多,則越少之磺胺二甲嘧啶酵素結合體能與抗體結合,經呈色反應後呈色越淡;反之則呈色越深。本分析方法為一快速、敏感且便於執行之方法,80個血清樣品能在2小時內分析完畢。

材料與方法

一、磺胺二甲嘧啶直接酵素連結免疫吸附劑分析套組(sulfamethazine one-step ELISA Kit)

購自International Diagnostic Systems Corp., U.S.A.(簡稱為IDS ELISA試劑套組),包括以下組成:

(一)塗附抗體微量測試孔(antibody coated removal-wells):

包括微量測試孔(microtiter well),每盒8條,每條12個微量測試孔,每個測試孔塗附有對磺胺二甲嘧啶高親合性抗體。

(二)磺胺二甲嘧啶酵素結合體(sulfamethazine enzyme conjugate)1瓶15 ml,為磺胺二甲嘧啶—蔞菜過氧化酵素結合體(sulfamethazine-horseradish peroxidase conjugate)。

(三)樣品及標準稀釋液(sample & standard diluent):375 ml (3只125 ml瓶裝),含0.1%牛血清白蛋白(bovine serum albumin, BSA)之磷酸鹽緩衝液。

(四)洗滌液(wash solution):

375 ml (3只125 ml瓶裝),磷酸鹽緩衝液中含Tween-20(非離子性界面活性劑)。

(五)陽性對照用標準液(positive control):

每瓶5 ml,共三瓶,含1000 ppm,1 ppm和0.001 ppm(1 ppb)三種濃度磺胺二甲嘧啶。

(六)基質溶液A(substrate solution A):

10 ml 3,3',5,5'-tetramethylbenzidine (TMB)。

(七)基質溶液B(substrate solution B):

10 ml 0.02%過氧化氫溶液。

(八)停止液(stop solution):

20 ml (2只10 ml瓶裝),1M H₃PO₄ (pH=3)

二、設備

(一)酵素免疫分析閱讀儀(enzyme immunoassay reader, EIA Reader):具有450nm波長者。

(二)其它必備器材:

平板振盪器、離心機、定量分注器、微量自動吸管(12孔及單孔)、吸管尖頭、洗滌瓶、定量瓶、注射筒、採血針、燒杯、吸管、試管、試管架。

三、實驗方法:

(一)樣品檢液之製備:

自豬頭靜脈採血約5 ml,俟凝固後離心分離出

血清,取0.2 ml血清加入於含1.8 ml稀釋液(含0.1 %牛血清白蛋白之磷酸鹽緩衝液)之試管中,於振盪器振盪混合後即得稀釋10倍之樣品液。

(二)標準液之製備:

精秤SMT標準品(Sigma Chemical Co.,USA, Catalog No. S-6256)25mg於定量瓶,以甲醇溶解並定量至25 ml,其濃度為1000 $\mu\text{g}/\text{ml}$ 作為原液。吸取0.1 ml原液於定量瓶中,以稀釋液定量至100 ml,其濃度為1.0 $\mu\text{g}/\text{ml}$ 當作中間溶液,吸取0.1 ml中間溶液於定量瓶中,以稀釋液定量至100 ml,其濃度為0.001 $\mu\text{g}/\text{ml}$ 。

(三)空白血清之取得及空白檢液之製備:

1.採用本測定法作測試時,選取吸光值高於或接近空白稀釋液或高於含SMT標準液0.001 $\mu\text{g}/\text{ml}$ 之血清當作空白樣品。

2.採用化學分析法進行血清樣品萃取後,再以TLC法作定性分析,確認無SMT殘留者,可當作空白血清樣品。

將空白血清樣品依上述1.法調製成空白血清檢液。

(四)陽性對照血清之製備:

1.含0.04 $\mu\text{g}/\text{ml}$ SMT之陽性血清:精確吸取空白血清2.0 ml置於試管中,添加1.0 $\mu\text{g}/\text{ml}$ SMT標準液85 μl ,振盪混合即可。此含0.04 $\mu\text{g}/\text{ml}$ SMT之血清相當於肌肉中SMT含量0.01 ppm,其互換公式為:

肌肉中SMT濃度=0.24x血清中SMT濃度⁽³⁷⁾。

2.含0.2 $\mu\text{g}/\text{ml}$ SMT之陽性血清:精確吸取空白血清2.0 ml置於試管中,添加1.0 $\mu\text{g}/\text{ml}$ SMT標準液0.5 ml振盪混勻即成。此含0.2 $\mu\text{g}/\text{ml}$ STM之血清相當於肌肉中SMT含量0.05 ppm。

3.含0.42 $\mu\text{g}/\text{ml}$ SMT之陽性血清:精確吸取空白血清2.0 ml置於試管中,添加1.0 $\mu\text{g}/\text{ml}$ SMT標準液1.45 ml振盪混勻即成。此含0.42 $\mu\text{g}/\text{ml}$ SMT之血清相當於肌肉中SMT含量0.1 ppm。再依上述1.樣品液之製備,分別調製成陽性血清檢液(1),(2)和(3)。

(五)測試方法:

將IDS ELISA試劑套組自冰箱取出,於室溫下回溫至少30鐘,使用前溫和地倒轉混合均勻。將微量測試孔條嵌入固定盤架上。依微量測試孔順序注入空白血清檢液20 μl 當作陰性對照;供對照用陽性血清檢液各20 μl ;待測血清檢液各20 μl 。每孔內均同時加入磺胺二甲嘧啶-萘菜過氧化酵素結合體液100 μl ,於振盪器上振盪混合10分鐘,使用清洗器以清洗液沖洗至少三次後拍乾,每孔內均同

時加入酵素基質溶液(A液和B液等量混合)150 μl 於平板振盪器上振盪反應10分鐘,立刻加入停止液(1M H_3PO_4 ,pH 3)混勻後以波長450nm之EIA Reader閱讀。

(六)判定和說明:

1.合格,SMT未檢出:血清樣品之ELISA檢測吸光值高於陽性血清(1)即0.04 $\mu\text{g}/\text{ml}$ 之吸光值,表示肌肉中無SMT殘留,因為肌肉中SMT殘留以TLC法分析之最低檢出感度為0.01 ppm,相當於血清中SMT 0.04 ppm。

2.合格,SMT<0.05 ppm:血清樣品吸光值低於陽性血清(1)0.04 $\mu\text{g}/\text{ml}$ 但高於陽性血清(2)即0.2 $\mu\text{g}/\text{ml}$ 之吸光值,表示肌肉中SMT殘留量大於0.01 ppm但小於0.05 ppm。

3.不合格,SMT>0.05 ppm:血清樣品吸光值低於陽性血清(2)即0.2 $\mu\text{g}/\text{ml}$ 但高於陽性血清(3)即0.42 $\mu\text{g}/\text{ml}$ 之吸光值,表示肌肉中SMT殘留量大於0.05 ppm但小於0.1 ppm。

4.不合格,SMT>0.1 ppm:血清樣品吸光值低於陽性血清(3)即0.42 $\mu\text{g}/\text{ml}$,表示肌肉中SMT殘留量大於0.1 ppm。

結果與討論

一、磺胺二甲嘧啶酵素免疫分析法標準檢量線

以空白血清添加使含SMT 0.04、0.2及0.42 μg

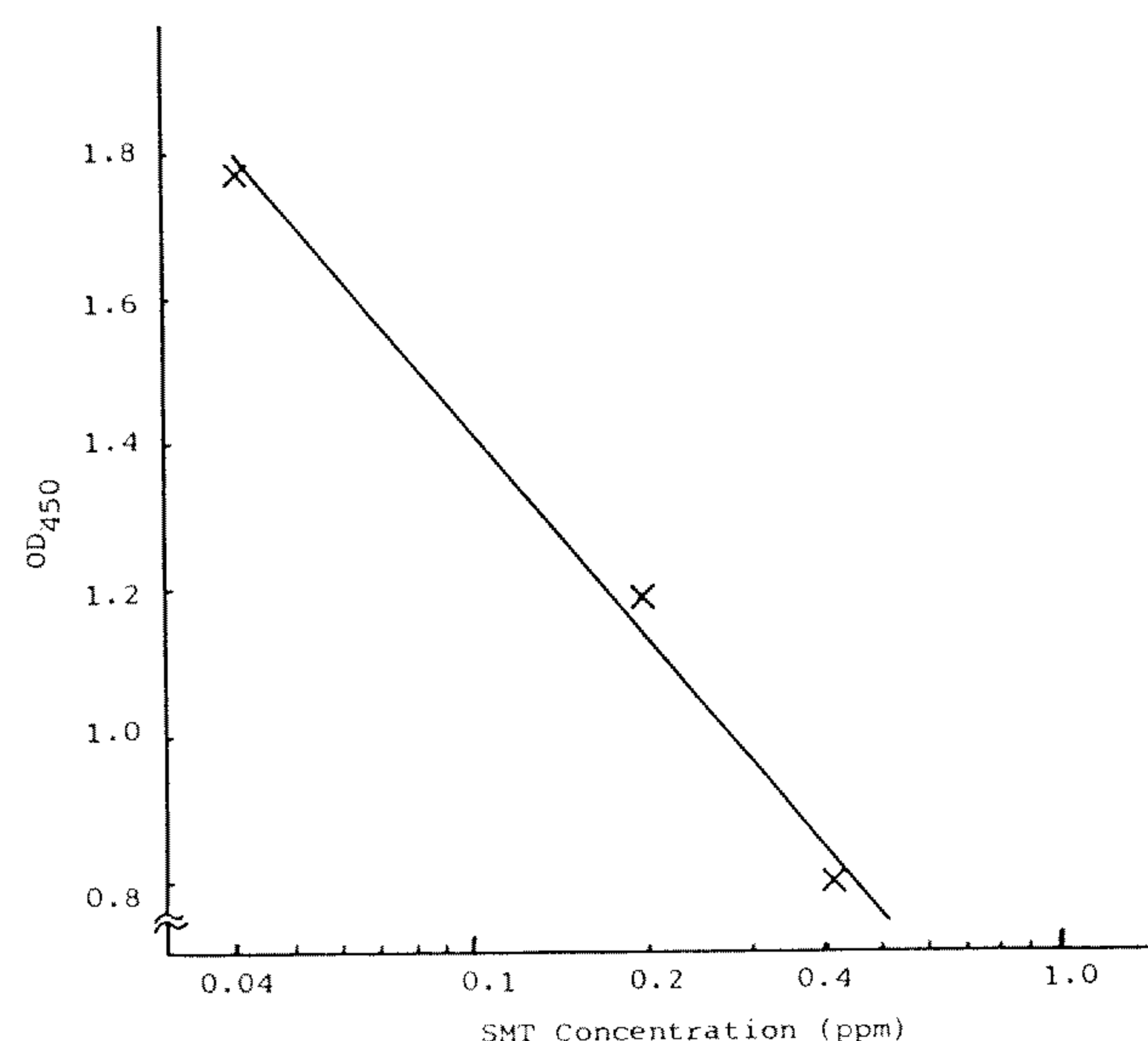


Figure 1. ELISA standard curve of sulfamethazine

/ml三種濃度和空白血清依上述實驗方法測試,包括空白血清及三種加藥空白血清各作六重複試驗,結果可得一線性良好之劑量—反應檢量線(linear dose-response curve),以吸光度對log [sulfamethazine]作圖如圖一,其相關係數為-0.9956, CV%為2.92%,表示磺胺二甲嘧啶在血清中濃度0.04-0.42 $\mu\text{g/ml}$ 之範圍間可準確地定量。

二、磺胺二甲嘧啶之回收率

方法的準確度可以回收實驗核對。於空白血清樣品中添加0.04,0.20和0.42 $\mu\text{g/ml}$ 磺胺二甲嘧啶作六重複試驗結果,磺胺二甲嘧啶回收率範圍為85.0-109.5%,平均值為99.8%(表一)。本法之回收率比HPLC法者為高,可能是因為HPLC法前處理步驟較繁⁽³³⁾,導致回收率較低。

三、SMT檢測專一性之探討

Table 1. Recovery of sulfamethazine determined by ELISA and HPLC

Fortification level(ppm)	Recovery by ELISA** (%)	Recovery by HPLC* (%)
0.04	109.5	92.0
0.20	104.9	89.4
0.42	85.0	87.4

*Data of Pan and Chen⁽³⁶⁾.

**The average of six determinations.

選取11種常用磺胺劑:sulfamethazine, sulfamerazine, sulfadimethoxine, sulfamonmethoxine, sulfathiazole, sulfamethoxypyridazine, sulfaquinoxaline, sulfamethoxazole, sulfapyridine, sulfisoxazole和sulfadiazine各四種不同濃度(即0.001,0.01,0.1和1.0 $\mu\text{g/ml}$)下進行交叉反應(cross-reactivity),假設50%抑制呈色反應值為具有交叉反應性之認定值⁽³⁷⁾,則其450nm之OD值B/B₀%為50%,當其他任何磺胺劑在各種不同濃度做測試而具有以上結果者,即可認為干擾成立,具有交叉反應。以下式計算交叉反應係數(cross-reactivity):

交叉反應係數=(SMT 50%結合之nanomoles/sulfa analogue 50%結合之nanomoles)×100%

以上述不同磺胺劑之四種不同濃度進行測試,結果示如表二。濃度在1.0 $\mu\text{g/ml}$ 時有sulfamerazine, sulfisoxazole和sulfapyridine等三種磺胺劑與抗磺胺劑抗體有交叉反應性。若以磺胺二甲嘧啶對抗磺胺二甲嘧啶抗體之交叉反應性為100%,則sulfamerazine為10.0%,sulfisoxazole為3.4%,sulfapyridine為2.0%。當上述三種磺胺劑濃度降低至0.1 $\mu\text{g/g}$ 時僅sulfamerazine仍具有1.6%之交叉反應性,其餘兩種在檢測上不會受到交叉反應性之影響。

四、磺胺二甲嘧啶直接酵素連結免疫吸附劑分析套組(IDS ELISA試劑)之偵測極限

SMT之ELISA檢測,在空白樣品中因無SMT存在故呈深黃色,當SMT存在時會依量之不同,呈色受不同程度之抑制,當設定20%抑制呈色反應

Table 2. Cross-reactivity of sulfamethazine antibody toward sulfamethazine analogues

Sulfamethazine analogues	% Cross-reactivity			
	0.001ppm	0.01ppm	0.1ppm	1.0ppm
Sulfamethazine	100	100	100	100
Sulfamerazine	0.03	0.06	1.60	10.0
Sulfadimethoxine	0	0.03	0.04	0.06
Sulfamonmethoxine	0	0	0	0
Sulfathiazole	0	0	0	0.07
Sulfamethoxypyridazine	0	0	0	0
Sulfaquinoxaline	0	0	0	0
Sulfamethoxazole	0	0	0	0
Sulfapyridine	0	0	0	2.0
Sulfisoxazole	0	0	0	3.4
Sulfadiazine	0	0	0	0

Table 3. Operation costs of ELISA and HPLC method for the analysis of sulfamethazine

Method	Time required (hr)	Assays per Person-day	Reagents cost outlay (NT\$)	Recovery (%)	Determination limit(ppm)
ELISA	0.025	320	50	99.8	0.001
HPLC	5	1.6	300-500	89.6	0.05

值做為SMT之檢測感度時,450nm OD值之B/Bo%為80%,此時SMT之濃度即為其偵測極限(detection limit)⁽³⁸⁾。換言之,若空白稀釋液之450 nm之OD值為1.500,則SMT之OD值為1.200時SMT之濃度即為其偵測極限。

由圖一知磺胺二甲嘧啶偵測極限為0.001 ppm(即1.00ppb),此值比日本之管制值低。

五、經濟效益分析

茲將ELISA和HPLC法定量磺胺二甲嘧啶之經濟效益比較示如表三。

由表三可知:在人力、耗費時間、費用、回收率以及偵測極限上,ELISA皆比HPLC法為佳,為一種極適用於豬體中磺胺二甲嘧啶檢測之方法。

結 論

IDS磺胺二甲嘧啶直接酵素連結免疫吸附劑分析試劑對磺胺二甲嘧啶有極佳之偵測極限(0.001 ppm)。又豬血清中殘留磺胺二甲嘧啶之含量為豬肌肉中四倍之多。因此比使用TLC法(偵測極限0.01 ppm)及HPLC法(偵測極限0.05 ppm)敏感50倍以上。由於血清樣品僅稀釋後即可測定,無需萃取、淨化及濃縮步驟,同時使用平板振盪器及高速酵素免疫分析閱讀儀,能在短時間內分析大量樣品,故為相當快速之SMT篩選法。近幾年來藉由融合瘤技術(hybridoma technique)生產單株抗體將更能提高檢測之專一性,降低與結構相似僅差一甲基基團之磺胺一甲嘧啶的交叉反應性。使用本酵素連結免疫吸附分析法可大幅縮短分析時間,達到快速篩選合格毛豬以供屠宰用。可將此法應用於養豬場之篩檢,經測試不合格的活毛豬繼續進行停藥飼養,待檢測合格後再送往屠宰場。由於日本官方公告之SMT殘留檢驗方法係採HPLC法,其豬肉分析之最低檢測感重為0.05 ppm,故對豬肉製品仍應以HPLC法作確認檢測,以達確認之管制目的。

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Determination of Sulfamethazine in Swine Serum with One-step ELISA Test Kit

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ABSTRACT

Sulfamethazine is a suspected carcinogen. It is widely used as feed additive for swine rations. An effective way of reducing this high incidence of sulfamethazine violations in swine would be to implement a nationwide slaughterhouse surveillance program for the drug. The traditional method for the detection of sulfamethazine spends too much time. A rapid screening test of sulfamethazine residue in swine serum was made by the use of the sulfamethazine one-stop Enzyme-Linked Immunosorbent Assay (ELISA) Kit. The time for sample preparation and reaction in microwells and read in ultrascan meter was about 30 minutes. The detection limit of sulfamethazine was 0.001 ppm. The mean recoveries of added sulfamethazine from serum at le-

vels 0.04, 0.2, and 0.42 $\mu\text{g/ml}$ were 99.8% (coefficient of variation, $\text{CV}=2.92\%$). Among 11 sulfonamide analogs: sulfamethazine, sulfamerazine, sulfadimethoxine, sulfamonomethoxine, sulfathiazole, sulfamethoxypyridazine, sulfaquinoxaline, sulfamethoxazole, sulfapyridine, sulfisoxazole and sulfadiazine with 4 different concentrations : 0.001, 0.01, 0.1 and 1.0 $\mu\text{g/ml}$ had cross reaction to antibodies in the ELISA Kit. The relative reactivity of sulfamethazine, sulfamerazine, sulfisoxazole and sulfapyridine were 100, 10.0, 3.4 and 2.0%, respectively. The concentration of sulfonamide below 0.1 $\mu\text{g/ml}$, the cross reaction was neglected. This ELISA kit may be used as a rapid screening test for sulfamethazine residue in swine serum.

Key Words : sulfamethazine, enzyme-linked immunosorbent assay (ELISA) kit, sulfonamide analogs, cross reaction.