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Establishment of a Fast Chemical Identification System for screening of counterfeit drugs of macrolide antibiotics

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Abstract

A Fast Chemical Identification System (FCIS) consisting of two colour reactions based on functional groups in molecules of macrolide antibiotics and two TLC methods was developed for screening of fake macrolide drugs. The active ingredients could be extracted from their oral preparations by absolute alcohol. Sulfuric acid reaction as a common reaction of macrolides was first used to distinguish the macrolides from other types of drugs and then 16-membered macrolides and 14-membered ones were distinguished by potassium permanganate reactions depending on the time of loss of colour in the test solution; after which a TLC method carried out on a GF_{254} plate (5 cm × 10 cm) was chosen to further identification of the macrolides. The mobile phase A consisting of ethyl acetate, hexane and ammonia (100:15:15, v/v) was used for the identification of 14-membered macrolides, and the mobile phase B consisting of trichloromethane, methanol and ammonia (100:5:1, v/v) was used for the identification of 16-membered ones. A suspected counterfeit macrolide preparation can be identified within 40 min. The system can be used under different conditions and has the virtues of robustness, simplicity and speed.

Keywords: Macrolide antibiotics; Counterfeit drug; Chemical identification system

1. Introduction

Spread of counterfeit drugs is a serious problem in countries all over the world, especially in developing countries. Although counterfeit drugs are part of the broader phenomenon of substandard pharmaceuticals, they are deliberately and fraudulently mislabelled with respect to identity and/or sources. Counterfeiting can be applied to both brand and generic products and counterfeit drugs may include products with the correct ingredients but fake packaging, with the wrong ingredients, without active ingredients or with insufficient active ingredients.

Since 1999, China was implementing a crackdown on the manufacturing and sales of counterfeit drugs, a problem which has been widespread on the mainland, especially in countryside [1]. However, finding counterfeit drugs is becoming a growing challenge in stopping the production and marketing of counterfeit medicines. Although simultaneous multi-component profiling and screening method in one-step employing LC–MS of high resolution, high sensitivity and definite structure determination would be more preferred for identification of counterfeit medicines, the methods reported in literature were more sophisticated [2,3] so that they can not be performed by laboratories that are not well equipped.

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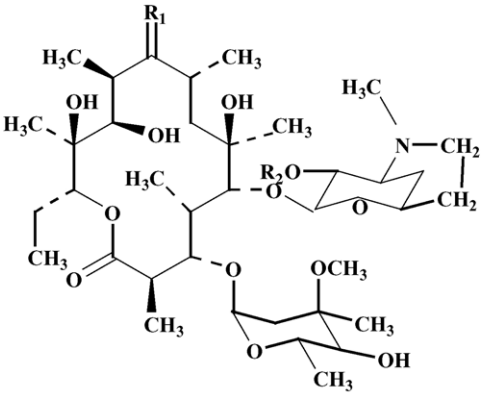
There is an urgent need for rapid and simple identification procedures for quick inspection of medicines in countryside and fighting against fake drugs. For this purpose, a "Fast Drug Identification System" which including a NIR pre-screening identification system and a fast chemical identification system equipped in a mobile vehicle is being developed in China and gradually put into use from 2005. About 400 categories of chemical medicines frequently used in China have been classified by their molecular structures, including agents for antipyretic-analgesic, anti-inflammatory, cardiovascular system, respiratory system, digestive system, nervous system, adrenocortical hormones, vitamins and micronutrients, anti-allergic and anti-infection etc., consequently different kinds of fast and specific chemical identification methods relevant to different molecular structures are being developed.

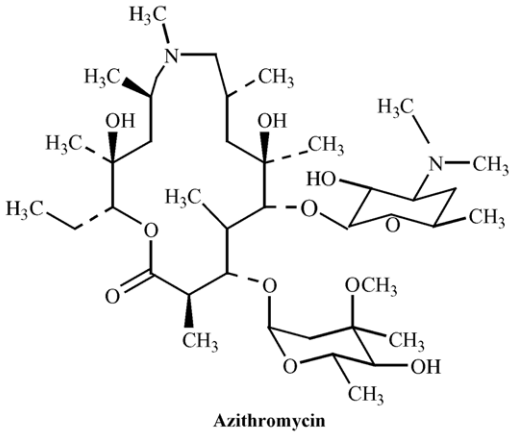
Macrolides are one of the widely used oral antibiotics in China. In recent years, some counterfeit drugs (without active ingredients or with a wrong ingredients, i.e. using erythromycin instead of roxithromycin) were found. In pharmacopoeias, bioassay and high performance liquid chromatographic (HPLC) methods are usually used for assay of macrolide antibiotics and thin-layer chromatographic (TLC) methods for identification [4–7]. There is scientific literature on separation and identification of the antibiotics by LC–MS [8–11]. However, few reports mentioned on systematic identification of macrolides using fast and special chemical methods, which have the virtues of simplicity, fastness and easy-to-operate. In our fast chemical identification system, 10 macrolide antibiotics in different preparations including erythromycin, roxithromycin, clarithromycin, azithromycin, erythromycin ethylsuccinate, midecamycin



Component	R_1	R_2	R_3
Erythromycin	=O	H	H
Roxithromycin	$=NOCH_2OCH_2CH_2OCH3$	H	H
Clarithromycin	=O	H	CH_3
Erythromycin Ethylsuccinate	=O	$-COCH_2CH_2COOCH_2CH_3$	H

Erythromycin derivatives

(a)



Azithromycin

(b)

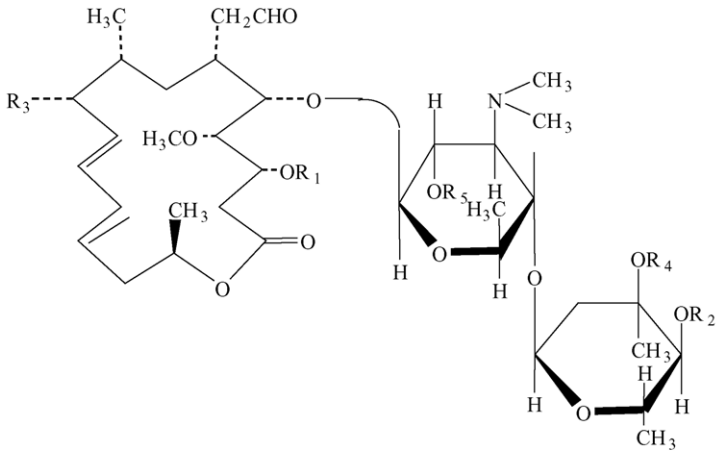
Fig. 1. Component structures of 14-membered macrolide antibiotics; (a) erythromycin derivatives; (b) azithromycin.

(major component is midecamycin A_1), meleumycin (major components are midecamycin A_1 and leucomycin A_6), kitasamycin (major component is leucomycin A_5), acetyl-kitasamycin (major component is acetylleucomycin A_4, A_5) and acetylspiramycin (contains four major components) (Figs. 1 and 2) can be first classified into two types: 14-membered and 16-membered macrolides according to their structures by two wet chemical tests, Then one specific TLC method developed on the basis of analogue molecular structures was chosen to distinguish the homologous drugs each other. A suspected counterfeit macrolide preparation can be identified using the identification system within 40 min.

2. Experimental

2.1. Materials

Erythromycin RS, clarithromycin RS, roxithromycin RS, azithromycin RS, erythromycin ethylsuccinate RS, kitasamycin RS, luecomycin A_3 RS, acetylspiramycin RS, acetyl-kitasamycin RS, midecamycin RS and meleumycin RS were obtained from National Institute for the Control of Pharmaceutical and Biological Products (NICPBP). Each oral preparation of the macrolides including tablets, capsules, granules and suspensions were produced from at least five different domestic pharmaceutical manufacturers and more



Type	Component	R_1	R_2	R_3	R_4	R_5
Leucomycins	leucomycin A_1	H	$COCH_2CH(CH_3)_2$	-OH	H	H
	leucomycin A_3	$COCH_3$	$COCH_2CH(CH_3)_2$	-OH	H	H
	leucomycin A_4	$COCH_3$	$COCH_2CH_2CH_3$	-OH	H	H
	leucomycin A_5	H	$COCH_2CH_2CH_3$	-OH	H	H
	leucomycin A_6	$COCH_3$	$COCH_2CH_3$	-OH	H	H
	leucomycin A_7	H	$COCH_2CH_3$	-OH	H	H
	leucomycin A_8	$COCH_3$	$COCH_3$	-OH	H	H
	leucomycin A_9	H	$COCH_3$	-OH	H	H
	leucomycin A_{13}	H	$COCH_2CH_2CH_2CH_2CH_3$	-OH	H	H
	midecamycin A_1	$COCH_2CH_3$	$COCH_2CH_3$	-OH	H	H
	acetylleucomycinA_1,A_3	$COCH_3$	$COCH_2CH(CH_3)_2$	$COCH_3$	H	$COCH_3$
	acetylleucomycinA_4,A_5	$COCH_3$	$COCH_2CH_2CH_3$	$COCH_3$	H	$COCH_3$
Spiramycins	3", 4"-diacetylspiramycin III	$COCH_2CH_3$	$COCH_3$	R'	$COCH_3$	H
	3", 4"-diacetylspiramycin II	$COCH_3$	$COCH_3$	R'	$COCH_3$	H
	4"-acetylspiramycin III	$COCH_2CH_3$	$COCH_3$	R'	H	H
	4"-acetylspiramycin II	$COCH_3$	$COCH_3$	R'	H	H

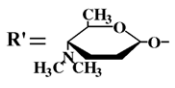


Fig. 2. Component structure of 16-membered macrolide antibiotics.

then ten lots of each preparation were obtained, which comply with the specification of Chinese Pharmacopoeia Version 2000. Six lots of counterfeit drugs, found in Chinese markets in recent years, were obtained from NICPBP.

Potassium permanganate, sulfuric acid, ammonia, absolute alcohol, methanol, acetone, trichloromethane, ethyl acetate, hexane, and iodine were of analytical grade bought from Beijing Chemical Reagents Co., China.

TLC silica GF_{254} plats (5 cm × 10 cm) were the product of Tianjin Si-Li-Da chromatographic technology Co., China.

2.2. Test solution preparation

Tablets, granules, and dry suspensions were first ground into fine powder. A quantity of the fine powder or the contents of capsules were dissolved in absolute alcohol, and shaken for about 3 min to produce a solution of about 25 mg of active ingredients per millilitre according to the label amount of the oral preparations. The solution was sedimented for about 5 min and the supernatants was taken as test solution.

Tens of same preparation produced by different manufacturers can be found in the market, as the macrolide antibiotics are very popular in China. To develop the optimum extraction method for both the colour reactions and the TLC tests, different conditions including solvents (methanol, absolute alcohol and acetone), extraction time and the concentrations etc were examined. For calculating the recovery, a quantity of reference standard was dissolved in methanol to produce a series of reference solutions of about 25, 20 and 15 mg/ml, the concentration of each extraction solution was estimated by comparing the intense of the spot corresponding to the major component of the macrolide with the series of the reference solutions on the TLC chromatogram, and then the recovery was calculated according to the label amount of the preparations. The recoveries of the extraction method mentioned above were at least 70% for all oral preparations and products produced by different manufacturers.

2.3. Colour reaction

2.3.1. Sulfuric acid reaction

To 1 ml of test solution, five drops of sulfuric acid solution (95–98%, w/v) were added and mixed well, and then the colour change of the solution after 1 min was observed.

2.3.2. Potassium permanganate reaction

To 1 ml of test solution, one drop of potassium permanganate solution (0.02 mol L^{-1}) [4] were added and mixed well, then the colour change was immediately observed and the time of loss of colour of potassium permanganate in the test solution was recorded.

2.4. TLC identification

The identification was carried out on a GF_{254} plate (5 cm × 10 cm). The mobile phase A consisted of ethyl acetate, hexane and ammonia (100:15:15, v/v). The mobile phase B consisted of trichloromethane, methanol and ammonia (100:5:1, v/v). A quantity of reference standard was dissolved in methanol to produce a reference solution of about 25 mg/ml. Except clarithromycin identification which used 4 μl of each of the solutions, 2 μl each of the reference solution and the test one were sampled to the plate. After developing, the plate was dried in the air, and then put in a closed tank with a quantity of iodine for several minutes until the spots appeared. When identification with mobile phase A, roxithromycin RS solution was sampled at the same time to check whether its R_f value was about in the range 0.45–0.65, which means that the TLC system is suitable for the determination. When identification with mobile phase B, midecamycin RS should be used to check weather the R_f value of midecamycin A_1 is about in the range 0.50–0.70.

3. Results and discussion

3.1. Colour reactions

Sulfuric acid reaction is widely used to produce a colour solution, which could be measured by a colorimetric assay method, in dissolution tests of macrolide oral preparations in Chinese Pharmacopoeia Version 2000 [4]. So, it is thought as a common reaction of macrolides. Although the mechanism of the reaction is not well known, except clarithromycin which appears a slightly brownish-red colour, all the macrolides tested in our experiments appear to brown or dark green colour in the reaction (Table 1) and all the preparations of macrolides tested had a similar colour change to its reference standard. The counterfeit drugs (without active ingredients) had no colour change, and other kinds of drugs had different colour change than the macrolides. For example, quinolones appear to slightly yellow colour, tetracyclines to a slightly dark yellow colour, sulfanilamide to a very slightly

Table 1
Phenomena of the colour reactions of macrolide antibiotics

Antibiotics	Colour in sulfuric acid reaction	Time of loss of colour in potassium permanganate reaction
Fourteen-membered macrolides		
Clarithromycin	Slightly brownish-red	6–11 min
Roxithromycin	Dark green	1–7 min
Erythromycin	Dark green	2–3 min
Erythromycin ethylsuccinate	Dark brown	2–3 min
Azithromycin	Dark green	∼1 min
Sixteen-membered macrolides		
Acetylspiramycin	Brown	12–14 s
Acetylkitasamycin	Dark brown	12–14 s
Kitasamycin	Dark brown	4–8 s
Midecamycin	Dark brown	4–6 s
Meleumycin	Dark brown	3–4 s

yellow colour, and steroids to a slightly pink-to-pink colour, etc. They can be easily distinguished from macrolides.

As a strong oxidant, potassium permanganate is easily reduced and fades in colour in the process. As shown in Figs. 1 and 2, in comparison with the 14-membered macrolides, the 16-membered macrolides mentioned in the paper belong to leucomycin and spiramycin families and exhibit a diene moiety, so that the molecules are more easily oxidized than the 14-membered ones. In the potassium permanganate reaction, all the 16-membered macrolides, either the reference standards or the preparations can cause the potassium permanganate solution dropped in the test solution to fade colour in less than 30 s at 10–30 °C, however, all the 14-membered ones usually need >1 min (Table 1). According to these phenomena, 16-membered macrolides can be easily distinguished from 14-membered ones.

3.2. TLC identification

The TLC tests were carried out in different regions of China at different seasons. Summarizing all the experiments, the TLC tests can be performed at 35–75% of relative humidity and 18–30 °C and need about 15 min for development. So, the methods are suitable for large scale applications for screening counterfeit drugs.

3.2.1. Fourteen-membered macrolides

The mobile phase A was used to identify the 14-membered macrolides (Table 2). In the TLC system, erythromycin and azithromycin could be distinguished easily according to the R_f values. However, the R_f value of erythromycin ethylsuccinate was similar to that of roxithromycin, and the roxithromycin's was similar to the clarithromycin's. As clarithromycin and roxithromycin have different colour changes in the sulfuric acid reaction (Table 1), they are not mistaken. However, erythromycin ethylsuccinate and roxithromycin have the similar colour changes. For distinguishing both of them, a hydrolysis test solution of erythromycin ethylsuccinate was needed, which produced by adding one drop of dilute hydrochloric acid (about 10%, v/v) to 1 ml of the test solution, mixed well for 2 min. Then both the hydrolyzed test solution and unhydrolyzed one were applied separately to a TLC plate. The relative R_f value of the hydrolyzed erythromycin ethylsuccinate to unhydrolyzed one is about 1.2, which well distinguishes erythromycin ethylsuccinate from roxithromycin.

3.2.2. Sixteen-membered macrolides

Not like the 14-membered ones, 16-membered macrolides usually contain multi-components. In China, midecamycin contains mainly midecamycin A_1, however meleumycin usually contains about 50% of midecamycin A_1 and about 15% of leucomycin A_6 [12]; kitasamycin contains at least nine leucomycin components (A_1, A_3, A_4, A_5, A_6, A_7, A_8, A_9 and A_{13}) and the main component, leucomycin A_5 is in 30–70% [13]; acetylspiramycin contains four main components and can be divided into 3″,4″-diacetylspiramycins and 4″-acetylspiramycins [4,5,13].

The mobile phase B was used to identify the 16-membered macrolides (Table 3). Five the different macrolide antibiotics could be distinguished by the R_f values in combining with the spot number and shape. In TLC system, meleumycin showed a principal spot of midecamycin A_1 with an R_f value of about 0.62 and a dark spot of leucomycin A_6 with an R_f value of about 0.36, which made it easy distinction from midecamycin, which only showed a principal spot of midecamycin A_1. Another spot with an R_f value of about 0.76 could be found in both midecamycin tablets and meleumycin tablets, and was thought to be an excipient. Kitasamycin showed an ellipse principal spot with an R_f value of about 0.64 and a very dark spot with an R_f value of about 0.44. As the R_f value of luecomycin A_3 RS was about 0.62, the ellipse spot was thought to be a mixture of A_5, A_4, A_1 and A_3 according to their structure (Fig. 1) and the RP–HPLC retention behaviour [8]. Acetylspiramycin showed two tailed principal spots with R_f values of about 0.74 and 0.33, and it was thought that they were mixtures of 3″,4″-diacetylspiramycins and 4″-acetylspiramycins. Acetylkitasamycin showed only one principal spot with an R_f value of about 0.86.

3.2.3. Influence of the variability on the method

All the TLC experiments were carried out over at least 15 months at five laboratories in different geographic regions of China. The operation circumstances covered 25–75% of relative humidity and 18–30 °C. Analysis of the influence of the operation condition on the method: the developing time was mainly affected by the temperature, and the R_f value mainly

Table 2
R_f values of 14-membered macrolides with mobile phase A from different laboratories ($n = 5$)

Laboratory	Erythromycin	Erythromycin ethylsuccinate	Roxithromycin	Clarithromycin	Azithromycin
1	0.41	0.47	0.50	0.52	0.63
2	0.49	0.53	0.65	0.68	0.70
3	0.47	0.59	0.55	0.57	0.71
4	0.51	0.57	0.58	0.61	0.65
5	0.48	0.52	0.53	0.55	0.58
Mean	0.47	0.54	0.56	0.59	0.65
S.D.	0.0377	0.0467	0.0572	0.0619	0.0532

Table 3
R_f values of 16-membered macrolides with mobile phase B from different laboratories ($n = 5$)

Laboratory	Midecamycin A_1	Luecomycin A_6	Kitasamycin		Acetylkitasamycin	Acetylspiramycin	
1	0.65	0.41	0.71	0.41	0.91	0.67	0.44
2	0.59	0.32	0.65	0.42	0.75	0.75	0.28
3	0.64	0.36	0.55	0.48	ND	0.74	0.34
4	0.67	0.45	0.60	0.46	ND	0.78	0.30
5	0.54	0.28	0.65	0.45	0.86	0.76	0.30
Mean	0.62	0.36	0.64	0.44	0.86	0.74	0.33
S.D.	0.0626	0.0680	0.0602	0.0303	0.0778	0.0418	0.0642

ND: not detected.

by the relative humidity. Usually, the R_f value reduced at a dry circumstance. For example, the relative humidity was usually less than 30% in winter in Xinjiang province, northwest of China, which made the R_f values of roxithromycin obtained from the system suitability test reducing to about 0.3–0.4, so that the 14-membered macrolides got a bad separation on the TLC chromatograms.

The interaction between the active pharmaceutical ingredient (API) and the excipient played another important role in influence on the R_f value. The oral preparations produced by different manufacturer even containing same API may have different excipients, which made a great variability for the R_f values. For example, the intermediate S.D. of roxithromycin R_f value obtained from system suitability tests under controlled circumstances was about 0.01. However, the S.D. value obtained from different roxithromycin preparations at the same condition became to about 0.03.

Although the R_f values in different conditions are not very reproducible, the sequence of all macrolides in the TLC chromatograms is not changeable. So, not only the absolute R_f value, but sometimes the ΔR_f value or the relative R_f value to the reference solution can be used to judge a counterfeit drug.

4. Identification system

An identification system for screening of fake drugs of macrolide oral preparations was established by combining both the colour reaction and the TLC test, by which 10 macrolides widely used in China can be quickly distinguished (Fig. 3). The identification system gave correct results for the fake drugs found in China, two lots of capsules and one lot of granule without any active ingredients imitating erythromycin ethylsuccinate capsule and azithromycin gran-

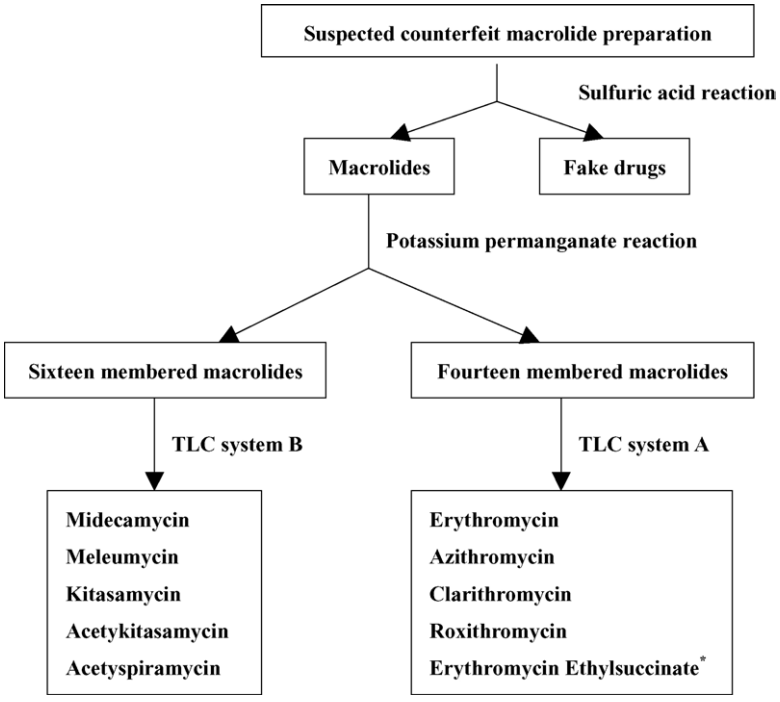


Fig. 3. Identification system for screening of fake drugs of macrolide oral preparations. Asterisk (*) denotes the hydrolyzed test solution and the normal one were applied at the same time.

ule, respectively, one lot of erythromycin tablets imitating roxithromycin tablets and two lots of meleumycin capsule imitating medicamycin capsule.

The identification system is suitable for a rapid screening of scene. It is very useful for finding fake drugs without active ingredients. However, when for finding counterfeit drugs with wrong ingredients, in case of a positive result the identification system can not be sufficient to identify a substance, an independent method, i.e. HPLC or LC–MS method to confirm the identity should be used later in a laboratory.

5. Conclusion

The identification system consists of colour reactions based on special functional groups in molecular structure of active ingredients and TLC methods can be used for screening of fake drugs of macrolide preparations widely used in China. The system has a good robustness for using in different conditions and has virtues of simplicity, fastness and easy-to-operate. The "Fast Chemical Identification System" equipped in the mobile vehicle for quick inspection of medicines in countryside and fighting against fake drugs in China has been adopted.

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