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Oxidative Demethylation of Dextromethorphan in Healthy Niger Delta Subjects by Thin layer chromatography

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A – research concept and design; B – collection and/or assembly of data; C – data analysis and interpretation; D – writing the article; E – critical revision of the article; F – final approval of article.

Abstract

Background: Classifying populations with a deficient capacity for oxidative drug metabolism is of increasing clinical significance. Dextromethorphan (DMP) is widely accepted as a probe drug to characterise individual variant expression of a specific cytochrome P-450 isozyme, *CYP2D6*. The thin layer chromatography (TLC) technique described in the present study is a rapid and inexpensive alternative to the methods currently available for assessing the urinary metabolic profile of DMP.

Objectives: To study the polymorphism of *CYP2D6* with DMP as probe drug by thin layer chromatographic method and to categorise the different *CYP2D6* phenotypes in a few subjects from the Niger Delta region of Nigeria.

Materials and Methods: Thirty-one healthy volunteers participated in the study by ingesting 30mg of DMP and collecting all urine for the ensuing 8h. Urine samples were analysed by TLC after treatment by acid hydrolysis. Phenotype assessment was based on the relative colour intensities of DMP and its O-demethylated metabolite dextrorphan (DRP).

Results: A greater intensity of the parent drug DMP relative to the metabolite DP indicates a poor metaboliser (PM) phenotype equal intensity an intermediate metaboliser (IM), whereas a reversed relative intensity whereby the intensity of DRP is higher indicates the extensive metaboliser (EM) phenotype and no spot seen indicating a possible ultrarapid metaboliser (UM). From the intensity of the spot on TLC, phenotypes were assigned such that 3.2% (1) of the individuals were PM, 12.9% (4) IM, 77.4% (24) EM and lastly 6.5% (2) UM.

Conclusion: The routine utilization of phenotype characterization into clinical protocols can be realized with this simple TLC technique.

Keywords: Genetic Polymorphism, Phenotyping, Dextromethorphan, Thin Layer Chromatography

INTRODUCTION

Cytochrome P-450 2D6 (CYP2D6) is responsible for the metabolism of 20-25% of clinically useful drugs (Chen *et al*., 2017). Consequently, polymorphisms on the CYP2D6 gene have the potential to affect efficacy, drug-drug interactions, and adverse events (Yang *et al*., 2017). Genetic polymorphism studies provide us with the ability to predict inter-individual differences in drug metabolism especially because of varying disease conditions (Yang, *et al*., 2017). Although several factors such as, age, sex, weight, disease conditions affect drug metabolism, genetic variations on genes encoding for drug metabolising enzymes, drug receptors, and drug transporters are the major determinants of interethnic and interindividual variability in the pharmacological or clinical response to drugs (Kim *et al* .,2004; Dodgen *et al*., 2013). For instance, the same dose of a drug which produces therapeutic effect in some patients may lead to adverse reactions and toxicity in others,

while in others may not produce sustained therapeutic effects (Qiao *et al*., 2016).

Dextromethorphan (DMP) is an innocuous antitussive drug found as one of the active ingredients in many over-the-counter cold and cough medicines and has also found other uses in medicine, ranging from pain relief to psychological applications. It is sold in syrup, tablet, spray, and lozenge forms (Chen *et al*., 2017) . The drug can be orally administered and is widely available, allowing it to be exploited in research as a probe drug for oxidative phenotyping. A large number of drugs are metabolised in the body by the cytochrome P450 (CYP enzymes), which are highly polymorphic; CYP3A4, CYP2C19, CYP2D6 are a few examples of these enzymes involved in the biotransformation in the body (Gaedigk *et al*., 2012). Dextromethorphan is well absorbed by the digestive system and it does not bind to plasma proteins. The adverse effects of DMP are quite low though care has to be taken when overdose occurs. The analysis of DMP and its metabolites dextrophan (DRP), 3-methoxymorphinan(3-MEM) and 3-hydroxymorphinan(3-OHM) have been encountered in both clinical and forensic settings. Both DRP and MEM are demethylated to 3-hydroxymorphinan and excreted in urine as glucoronide conjugates (Chladek *et al*., 2000). However this study will focus on DMP and DRP because the cumulative metabolism of the other metabolites represents less than 15% of the dose administered in 8hrs (Chladek *et al*., 2000).The DMP/DRP metabolic ratio (MR) can be measured in urine, plasma and saliva by HPLC, GC or HPTLC methods (Basci *et al*., 1998, Chladek *et al*., 2000). The advantage of using urine arises mostly from the fact that it is obtained in large volumes hence there is room for enough repeatability and testing of samples. Subjects with metabolic ratios higher than 0.3 are considered to be PMs while those below are termed extensive metabolisers when results are quantitative in nature.

DMP is metabolised to the O-demethylated metabolite DRP via CYP2D6 enzyme pathway. The intensity and metabolic ratio of DMP and DRP reflects the CYP2D6 enzyme activity depending on the method used and can be used to classify an individual as either a poor (PM), intermediate (IM), extensive (EM) or ultrarapid (UM) metabolisers (Todor *et al*., 2017). The metabolism of a number of other drugs such as debrisoquine and sparteine is known to be affected by this same genetic polymorphism (Eichelbaum, 1982). The diminished oxidative biotransformation of these compounds in the poor metaboliser population can lead to excessive drug accumulation, increased peak levels, or in some cases, decreased generation of active metabolites or untoward effects from recommended doses of some compounds that rely on the deficient isozyme for biotransformation (Idle *et al*., 1983). However, routine clinical screening of subjects to identify this metabolic deficiency is not adequately performed .This may in part be due to the techniques presently available for this purpose. Due to the fact that the analytical methods currently used to characterise phenotypes using DMP are too cumbersome and time-consuming to be cost-effective measures in a clinical setting, it is a problem probing it for genetic screening (Kupfer *et al*., 1986).

The availability of a facile method for phenotype identification should encourage the incorporation of pharmacogenetics screening into drug development strategies and therapeutic protocols. The Thin-layer chromatography (TLC) procedure presented here represents the most simplified means to screen for this deficiency in oxidative metabolism. In addition to facilitating large scale screenings for pharmacogenetic studies of experimental interest and clinical studies (Jain, 2000). Thin layer chromatography is very efficient in that it can always come in handy when emergency results are needed for example in drug abuse or forensic cases. This study therefore sets out to evaluate the polymorphism of CYP2D6 with dextromethorphan as probe drug by thin layer chromatographic method and to categorise the different CYP2D6 phenotypes in the ethnic population of south-south Nigeria.

MATERIALS AND METHODS

MATERIALS

Reagents

Dextromethorphan hydrobromide (Zedex cough syrup), Dextromethorphan reference standard (Sigma Aldrich), Dextrorphan reference standard (Sigma), Dichloromethane (DCM), Ethyl acetate, Methanol (CH_3OH), Ammonium hydroxide (NH_4OH), Concentrated hydrochloric acid (HCl), 0.1N HCl, Distilled water, and Iodine

Apparatus used

Analytical weighing balance, TLC plates, TLC tank, Sample calibrated containers, 2L plastic containers, Heparinised capillary tubes, Centrifuge tubes, Centrifuge, Iodine tank, Water bath (thermostated), Pasteur pipettes, Beakers, Conical flasks, Volumetric flasks, Measuring cylinders, Test tube rack, Fume cupboard, Micropipettes, Surgical gloves, Burettes, Droppers, Human weighing scale and height measuring apparatus.

METHODS

Data collection instrument

A self administered questionnaire was developed containing 21 items grouped into the following sections:

Section A: Demographic data consisting of age, sex, marital status, ethnicity, parents' and grand-parents' ethnicity, weight, height

Section B: consisting of social history of the volunteer including: smoking, alcohol, herbal medication, taking of any contraceptives

Section C: This section involved information on any concurrent illness

Study population

Thirty-one healthy individuals aged 17 to 34 years comprising 14 males and 17 females were recruited for the study and gave informed consent. The pre-study screening was done by (interviewing both orally and filling a questionnaire providing vital information) and physical examination. A thorough screening process, which include physical examination and laboratory tests such as liver and kidney function tests were undertaken to ensure that subjects were healthy and to exclude presence of infections and use of the possible inhibitors of CYP2D6 and CYP3A4. Candidates were appropriately monitored at least a month prior to the date of the sample collection to ensure that they adhered to the conditions. None of the subjects had a history of alcohol addiction and drug abuse or dependence, and they did not have any medical condition that required treatment. The subjects were advised not to take any medication (e.g. natural remedy or over-the-counter drugs) two weeks before the test. They were also asked to refrain from alcohol intake one week prior to the intake of the study drug and were asked to refrain from ingesting products containing grapefruit throughout the study. All subjects were asked to report any intake of medication throughout the study. At the time of entering the study all subjects were assessed as healthy. The study was carried out according to the Declaration of Helsinki (2000) of the World Medical Association and was approved by the Ethical and research committee of the Niger Delta University.

Drug administration and sample collection

Prior to drug administration all the subjects fasted overnight and food intake was not allowed for a period of 3hrs following drug administration. .Food intake was not regulated thereafter.The study was performed using a parallel single dose design. The thirty-one (31) subjects were each administered with 30ml dextromethorphan hydrobromide syrup containing (10mg/10ml) after which they were encouraged to drink enough water.Thereafter all urine voided between 0-8h was collected after drug intake. The total volume of urine was measured immediately after collection. Aliquots of urine samples were taken into fresh calibrated urine containers and stored frozen at -20°C before analysis.

Preparation of standard solutions

Stock solution of dextromethorphan hydromide (1mg/ml)

10mg of dextromethorphan hydrobromide powder was carefully and accurately weighed in a sample bottle on an analytical balance and dissolved in 0.1N dilute HCl in a volumetric flask. Thereafter the solution was made up to a final volume of 10ml.

Stock solution of dextrorphan (1mg/ml)

The same process was repeated again but this time using 10mg dextrorphan powder instead.

Determination of DMP and DRP In Urine Using Acid Hydrolysis

A 1ml sample of urine was each placed in an extraction tube and concentrated HCl (300µL) was then added to each tube. The tubes were capped and vortexed and then heated for 1.5h at 100ºC. At the end of the incubation, the pH of the samples was raised to 11 with concentrated NH_4OH (1.4ml) after which dichloromethane (4ml) was added .The samples were then mixed properly and centrifuged .After centrifuging, the aqueous phase was aspirated off and the organic layer containing the drug was transferred to a fresh tube and evaporated to dryness. To the residue 2 drops of dicholoromethane (DCM) was added and the entire volume was spotted onto the origin of a thin layer plate. The plate was developed over a distance of 5.5cm in a modified Davidow solvent system explained below. The TLC plates were then viewed by placing in an iodine tank and the spots intensities noted as well as their R_f values calculated properly. At all instances the same concentration, duration and intensity of spraying (with iodine in the iodine tank), type of silica used were all kept constant to minimize errors.

Development of TLC mobile phase

The plate was developed over a distance of 5.5cm in a modified Davidow solvent system which was found to be compatible with the work. Development of the mobile phase was done in the following ratio: ethyl acetate: methanol: ammonium hydroxide 17:2:1vol/vol/vol (ml) in a TLC tank. After the solvent had successfully reached the solvent front it was carefully brought out of the tank, allowed to air dry. Thereafter the plate was placed in an iodine tank

for proper identification and viewing of intensity of spots. Drug and metabolite spots appeared brown, their respective intensities were noted and their R_f values properly calculated. Samples were spotted alongside the reference standard drugs for appropriate comparison and to lend credence to the results. Reference standards were also spotted separately on their own and the intensity and R_f values noted.

Thin layer chromatographic analysis for phenotype assessment

The criterion used for phenotype assignment with TLC is the relative colour intensities of dextromethorphan (DMP) and dextrorphan (DRP) in a given sample .This involved the virtual observation and determination of the colour intensities in the sample (Fig 1A and B). If the intensity of the DMP spot was greater than that of DRP spot, the subject was assigned a poor metaboliser (PM) phenotype. On the other hand, if the metabolite (DRP) spot was of equal intensity with the parent drug spot, the subject was deemed to be an intermediate metaboliser (IM), if the intensity of the DRP spot was higher than DMP the individual was seen to be an extensive metaboliser (EM) while if no spot of DMP and DRP was detected then the individual was termed an ultrarapid metaboliser (UM).

RESULTS

Demography

The demographic data of the study population is as shown in Table 1.

Table 1: Demographic data on the study population

CODE	AGE	SEX	W(kg)	HEIGHT(M)	ETHNICITY
1S	22	F	50	1.68	IJAW
2S	21	F	50	1.56	IJAW
3S	25	M	76	1.95	IGBO
4S	24	F	61	1.73	IJAW
5S	24	F	64	1.63	IGBO
6S	20	F	50	1.58	IJAW
7S	22	M	67	1.72	IGBO
8S	25	M	69	1.75	IJAW
9S	34	M	69	1.68	IJAW
10S	24	M	56	1.72	IGBO
11S	25	M	82	1.88	IGBO
12S	17	F	74	1.76	IGBO
13S	24	M	59	1.63	IJAW
14S	23	F	54	1.7	IGBO
15S	22	F	52	1.73	IJAW
16S	21	F	61	1.67	IJAW
17S	22	F	59	1.64	IJAW
18S	25	F	75	1.6	IJAW
19S	19	F	55	1.44	IJAW
20S	25	F	62	1.69	IGBO
21S	25	M	76	1.93	ISHAN
22S	28	F	48	1.68	IJAW
23S	29	M	70	1.67	IJAW
24S	24	M	72	1.72	IJAW
25S	22	M	64	1.64	IJAW
26S	29	M	60	1.6	ISHAN
27S	23	M	80	1.84	IJAW
28S	23	F	65	1.61	IG BO
29S	22	M	68	1.8	IJAW
30S	21	F	94	1.77	IJAW
31S	22	F	65	1.66	IJAW

Phenotype assignment

Based on the colour intensity of the spot on TLC, phenotypes were assigned such that 3.2% (1) of the subjects were poor metabolisers, 12.9% (4) intermediate metabolisers, 77.4% (24) extensive metabolisers and lastly 6.45% (2) ultrarapid metabolisers as shown in Fig 2. In the PMs the DMP spot was much larger and more intense than the corresponding DRP spot. The DRP spot derived from all EMs was more intense than the corresponding DMP spot. It was noted that for the IMs the intensity for both the parent drug and the metabolite was observed to be equal in all instances. In one of the samples there was equally light spot for both DRP and DMP while in the remaining 3 samples both spots were equally very intense. Out of the 24 EMs, it was only 2 of the samples that both DRP and DMP were seen with DRP been very intense in both cases. Of these 2 DMP gave a light spot in both of them. In the remaining 22 EMs the parent drug was not detected, only DRP was seen. Of these 7 showed light spots, 4 intense spots and 13 very intense spots. No spot for both DMP and DRP was observed in 2 of the samples implying UM phenotype (Table 2).

The R_f values of dextromethorphan ranged from 0.72 to 0.75 while that of dextrophan ranged from 0.56 to 0.68

A B

Fig 1 (A and B): Representative TLC plates depicting:

A. From left to right: Showing standard DMP, standard DRP and standard mixture
B. From left to right: Showing standard DMP, standard DRP, an IM, a UM, and 2 Ems

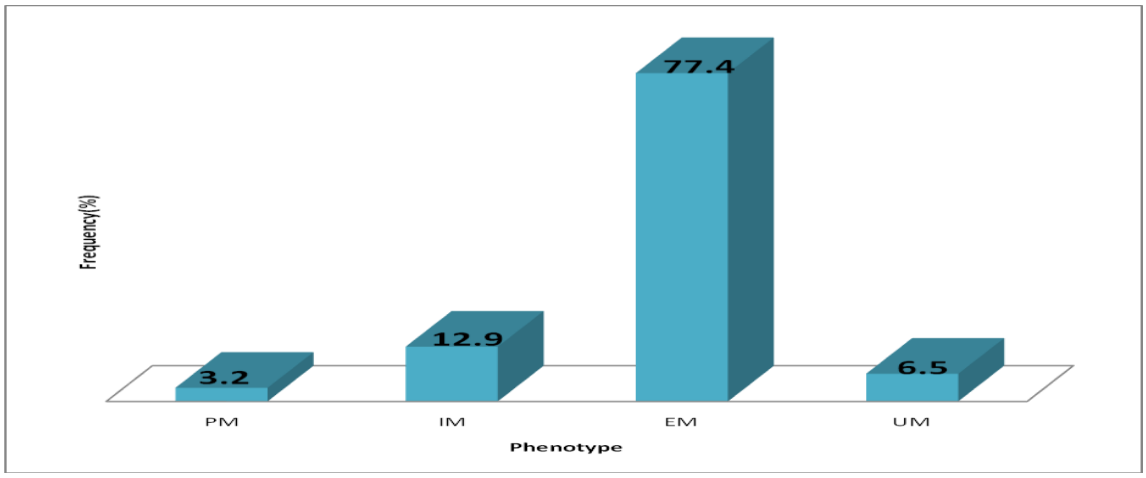

Fig 2: Bar chart showing percentage phenotype versus the different phenotypes

Table 2: TLC Results and Phenotype Assignment

CODE	INTENSITY	R_f VALUE		PHENOTYPE
		DRP	DMP	
1S	V	0.66		EM
2S	L	0.62		EM
3S	N S	N S	N S	UM
4S	L	0.62		EM
5S	V	0.6		EM
6S	V	0.65		EM
7S	V, I	0.6	0.74	EM
8S	L, L	0.61	0.77	IM
9S	L	0.57		EM
10S	V	0.6		EM
11S	V	0.62		EM
12S	N S	N S	N S	UM
13S	L, V	0.56	0.73	PM
14S	V	0.64		EM
15S	I, L	0.6	0.73	EM
16S	V	0.65		EM
17S	V, V	0.66	0.75	IM
18S	V, V	0.66	0.72	IM
19S	I	0.66		EM
20S	L	0.67		EM
21S	L	0.65		EM
22S	L	0.65		EM
23S	I	0.66		EM
24S	V	0.6		EM
25S	V	0.6		EM
26S	V	0.68		EM
27S	V	0.62		EM
28S	V	0.68		EM
29S	V, V	0.60	0.73	IM
30S	L	0.66		EM
31S	I	0.64		EM

Where, V= very intense spots I= intense spots L=light spots N S= no spot observed
EM=extensive metaboliser PM=poor metaboliser UM=ultrarapid metaboliser
IM=intermediate metaboliser
Standard of DMP= 0.75 Standard of DRP= 0.65

DISCUSSION

Pharmacogenetics-based studies is of special interest especially with drugs with narrow therapeutic indices that are substrates of polymorphic enzymes, where impairment in metabolic activity might cause difficulties in dose adjustment, resulting in increased susceptibility to adverse drug reactions. Dextromethorphan is widely used as a probe drug for CYP2D6 oxidation phenotyping in subjects from all over the world. Differences in metabolic rates exhibit substantial racial and interethnic variations (Samer *et al*., 2013).

In extensive metabolizers we have noted a large elimination of dextrorphan within the initial part of the urine collection whereas dextromethorphan has a clearly delayed elimination in the poor-rnetabolizer phenotype. Therefore, in extensive metabolizers cumulative urine collection in the first four hours is important, whereas the extended urine collection of up to eight hours is primarily relevant to poor metabolizers. It should be noted that these data are generated by means of cumulative drug and metabolite excretions (Kupfer *et al*., 1986). The percentage obtained for PMs which was 3.2% correlated closely with the 1-5% shown for PMs in the Nigerian population (Ebeshi *et al*., 2011; Matimba *et al*., 2008).This could probably have also been due to the small sample size used in this study or as a result of drug interactions which could have

slowed down the metabolism. However the result varies from studies carried out among Caucasians in which the study showed that 45 Turkish and 102 Greek yielded percentages of 6.9% and 6.7% respectively (Basci *et al*., 1988, Johnson *et al*., 2000). In PMs it is expected that there will be almost undetectable amounts of the metabolite in the presence of increased concentration of the unchanged parent drug in urine (Marinac *et al*., 1995). This could be due to a mutation on the CYP2D6 gene on chromosome 22(CYP2D6*3, CYP2D6*4, CYP2D6*5) associated with DMP metabolism, which would have prevented normal metabolism from taking place (Qiao *et al*., 2016). PMs inherit two deficient CYP2D6 alleles and, as a result, metabolize drugs at a notably slower rate. This leads to an accumulation of high levels of unmetabolized drugs that are CYP2D6 substrates, a concomitant greater potential for adverse events and drug–drug interactions, and lower efficacy for drugs requiring CYP2D6 activation. Care should be taken when administering these individual drugs as toxicity may occur. In the PMs it was noticed that the intensity of the DMP spots were higher than the intensity of the DRP spots. This implies that in this individual there was little or no CYP2D6 activity. A drug may also be less effective for a PM at CYP2D6 if the drug needs to be activated by CYP2D6. PMs usually require lower doses to achieve desired effects. The PM will accumulate the parent drug and incur the risk of enhanced effects. The drug may be secondarily metabolised by another cytochrome P450 enzyme that is higher in capacity but has a lower affinity for the drug or substrate. Often the alternative is CYP3A4. This shifting to a less efficient enzyme leads PMs to have higher drug levels of the parent compound (Eichold *et al*., 2007).

The intermediate metabolisers (IMs) phenotype, which accounts for the intermediate DMP metabolism between PMs and EMs. These are individuals in whom the intensity was equal for both DMP and DRP. Such individuals have their enzyme activity between poor and extensive metaboliser hence explaining the reason why their intensity was the same (In genotyping one functional allele and one mutant allele). Usually they need slight dose changes and are often even merged with extensive metabolisers as their response to drugs is not very different. There were instances where some spots of DMP and DRP were light and in other samples intense. There were actually no clear differences between the IMs and the EMs probably due to the small number of subjects and interindividual differences and this explains why a lot of times these 2 phenotypes are usually grouped together. This could have been due to a lot of factors affecting metabolism such as diet and genetic differences (Capron *et al*., 1996, Nelson *et al*., 2004, Aklillu *et al*., 2007).

A large percentage of the subjects in this study were found to be extensive metabolizers In these individuals the parent drug as already explained was not detected in most samples implying that it was of negligible importance. The metabolite was clearly detected varying from being light spots to very intense spots. These are individuals with normal CYP2D6 activity which will metabolise DMP at the normal rate than it should be metabolised. This high prevalence of EMs suggests that a greater proportion of the population metabolise drugs adequately at a normal extensive rate and this explains the fact why most pharmaceutical companies produce drugs bearing this particular group of people in mind. This data correlated with a study in Nigerian population in which a percentage of 79% were classified as EMs using the HPLC method (Ebeshi *et al*., 2011). In Caucasians there exist mainly two extremes of activity as either EMs or PMs While there exists a good proportion of IMs and EMs in the African population (Solus, 2004).

In the UMs no spot was detected and thus showing a clear demarcation from the EMs. The 6.45% obtained for this phenotype was however found to be slightly higher than what is obtainable in Blacks which is 4.9% with the exception of Ethiopians, which is 29% due to a change in their allele locus and ancestry with Arabians who have a percentage of 21% (Aklillu *et al*., 1996). This could be as a result of excess CYP2D6 activity which could have been caused by duplication and multiplication of genes that metabolised DMP at a very fast rate. In determining drug therapy for such individuals, doses of drugs need to be increased because the normal dosage will yield no effect.

The present study demonstrated that a significant savings in time and costs can be realised with the TLC technique. A group of 31 subjects can be efficiently screened by TLC in 4hrs if all necessary apparatus are enough and a single plate can accommodate as many samples as possible spaced 0.5cm apart. Quantitating MRs by HPLC in this same number of subjects could take 12 times more and would demand a considerable consumption of resources. Visual comparisons of TLC spot intensities and/or spot sizes have been used for rough, semiquantitative determinations in TLC for many years. In the present study, a less rigorous qualitative approach was employed that places emphasis on the relative intensities of the spots of interest rather than their actual quantitation. Based on this method, no serious difficulties were encountered when

characterising the apparent phenotype of any subjects participating in this study.

However, assigning an accurate phenotype may be complicated by the ability of certain drugs to inhibit CYP2D6 when coadministered with substrate drugs, because CYP2D6 is a low-capacity, high-affinity enzyme that is easily saturated by substrate. In such cases, metabolite formation of a probe drug may be reduced, potentially changing an individual's apparent phenotype from an EM to a PM (Clark, 1985, Lutz *et al*., 2004). The ability to screen for this genetic polymorphism in drug oxidation can have a profound positive impact on both the development and clinical usage of drugs. If polymorphic metabolism of a drug can be recognized during its development, dosing recommendations can be proposed, before its widespread use, which account for phenotypic differences in its handling. Additionally, if phenotypes are identified before the inception of therapy with drugs affected by this polymorphism, their clinical effectiveness might be improved and possible toxicity averted, in poor oxidative metabolisers.

The availability of a facile method for phenotype identification should encourage the incorporation of pharmacogenetic screening into drug development strategies and therapeutic protocols. The TLC procedure presented here represents the most simplified means available to screen for this deficiency in oxidative metabolism. In addition to facilitating large scale screenings for pharmacogenetic studies of experimental interest, this technique should also be attractive to those in the clinical area.

It can thus be concluded that phenotyping is a very useful method both for researching the genetic oxidation polymorphism and for individual treatment with drugs whose metabolism involves oxidative pathways. Thus it can be concluded that the use of TLC as phenotype identification can adequately be used in determining different metabolisers in their metabolism of dextromethorphan.

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