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(54) Title: ELISA KIT FOR THE DETERMINATION OF CYP 2C9 METABOLIC PHENOTYPES AND USES THEREOF

(57) Abstract: The invention relates to an enzyme linked immunosorbent assay (ELISA) method and kit for the rapid determination of metabolic phenotypes for Cytochrome P450 2C9 (CYP 2C9). The kit uses may include but are not limited to, use on a routine basis in a clinical laboratory to determine a Cytochrome P450 2C9 (CYP 2C9) phenotype of an individual; to allow a physician to individualize an individual's treatment with respect to the numerous drugs e.g. ibuprofen, losartan, E-3174 and 2-carboxyibuprofen metabolized by CYP 2C9 based on a phenotypic determination; to predict an individual's susceptibility to carcinogen induced diseases including many cancers, and to screen individuals for a preferred metabolic phenotype in order to determine those individuals with a responsive phenotype for participation in clinical testing.



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ELISA KIT FOR THE DETERMINATION OF CYP 2C9 METABOLIC
PHENOTYPES AND USES THEREOF

BACKGROUND OF THE INVENTION

(a) Field of the Invention

The invention relates to an enzyme linked immunosorbent assay (ELISA) method and kit for the rapid determination of metabolic phenotypes for Cytochrome P450 2C9 (CYP 2C9). The kit uses may
5 include but are not limited to, use on a routine basis in a clinical laboratory to determine a CYP 2C9-specific phenotype of an individual; to allow a physician to individualize an individual's treatment with respect to the numerous drugs metabolized by
10 CYP 2C9 based on a phenotypic characterization of the individual; to predict an individual's susceptibility to carcinogen induced diseases including many cancers, and to screen individuals for a preferred metabolic phenotype in order to determine those individuals with
15 a responsive phenotype for participation in clinical testing and/or for treatment with a particular drug or class of drug compounds.

(b) Description of the Prior Art

For the majority of drugs (or xenobiotics) administered to humans, their fate is to be metabolized in the liver, into a form less toxic and lipophilic with their subsequent excretion in the urine. Their metabolism involves two systems which act consecutively: the cytochrome P450 system which includes at least 20 enzymes catalyzing oxidation reactions and localized in the microsomal fraction, and the conjugation system which involves at least 5 enzymes. An enzyme of one system can act on several drugs and drug metabolites. The rate of metabolism of a drug differs between individuals and between ethnic groups, owing to the existence of enzymatic polymorphism within each system. As a result, a variety of phenotypes can be distinguished, including poor metabolizers (PM), extensive metabolizers (EM), and ultra-extensive metabolizers (UEM).

As described in U.S. Patent No. 5,830,672, Applicants have previously been successful in establishing an ELISA based system and method for the rapid determination of N-acetyltransferase (NAT2) phenotypes. However, to date a convenient and

effective system for determining CYP 2C9 phenotypes has not been provided.

In previous studies, CYP 2C9 phenotypes have been generally determined by determining the ratio of
5 the probe substrate (s)-ibuprofen and its metabolite 2-carboxyibuprofen in an individual . In these studies, the individuals ingest a dose of (s)-ibuprofen, and the urinary concentrations of the two compounds are determined by liquid chromatography/tandem mass
10 spectrometry (LC/MS/MS) or high-pressure liquid chromatography (HPLC). Existing CYP2C9 determination methods are time-consuming, onerous, and employ systems and equipment which are not readily available in a clinical laboratory.

15 It would be highly desirable to be provided with a convenient and effective method for characterizing an individual's CYP 2C9 phenotype using a non-toxic substrate so as to predict his/her response and side effects profile to a wide range of potentially toxic
20 drugs.

It would be highly desirable to be provided with an enzyme linked immunosorbent assay (ELISA) kit for CYP 2C9 phenotyping, which could be accomplished on a

routine basis by any technician with a minimum of training and does not involve complex equipment.

It would also be highly desirable to be provided with an enzyme linked immunosorbent assay (ELISA) kit, which would enable a physician to individualize therapy and/or treatment. Such therapies may include treatment with drugs such as phenytoin, tolbutamide, and nonsteroidal anti-inflammatory drugs (NSAIDS) based on an individual's CYP 2C9-specific phenotype.

10

SUMMARY OF THE INVENTION

One aim of the present invention is to provide an enzyme linked immunosorbent assay (ELISA) kit for the rapid determination of metabolic enzyme phenotype, which can be used on a routine basis in a clinical laboratory.

15

Another aim of the present invention is to provide an ELISA kit which allows a physician to:

- a) determine the CYP 2C9 metabolic phenotype of an individual;
- b) individualize therapies or treatments with drugs known to be dependent on CYP 2C9 metabolism, according to an individual's metabolic phenotype;

c) predict an individual's susceptibility to carcinogen induced diseases such as various cancers;

d) screen individuals for a preferred CYP 2C9 metabolic phenotype in order to determine those individuals with a responsive phenotype for participation in clinical testing.

Another aim of the present invention is to provide a method for determining an individual's metabolic enzyme phenotype in order to predict his/her responsiveness to a drug treatment regime.

5 The ELISA phenotyping kit according to an embodiment of the present invention employs at least one non-toxic substrate (or probe substrate) known to be metabolized by the CYP 2C9 pathway for the determination of the CYP 2C9 phenotypes.

10 According to one aspect of this invention there is provided a method of characterizing a CYP 2C9-specific phenotype, said method comprising (a) administering to an individual a substrate known to be metabolized by a CYP 2C9 metabolic pathway; (b)
15 detecting metabolites of said metabolic pathway in a biological sample obtained from the individual at a predetermined time after the administering of said

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substrate; and (c) characterizing a phenotypic determinant based on said metabolites which is indicative of said CYP 2C9 phenotype.

According to another aspect of this invention
5 there is provided a competitive enzyme linked immunosorbent assay (ELISA) method for determining a CYP 2C9 phenotype, which comprises using at least two antibodies specific to (s)-ibuprofen and 2-carboxyibuprofen respectively, to determine the amount
10 of each of (s)-ibuprofen and 2-carboxyibuprofen respectively, in a biological sample obtained from an individual treated with (s)-ibuprofen; wherein a molar ratio based on amounts of the (s)-ibuprofen to 2-carboxyibuprofen is indicative of a CYP 2C9 phenotype
15 of said individual.

According to another aspect of this invention there is provided a competitive enzyme linked immunosorbent assay (ELISA) method for determining a CYP 2C9 phenotype, which comprises using at least two
20 antibodies specific to losartan and E-3174 respectively, to determine the amount of each of losartan and E-3174 respectively, in a biological sample obtained from an individual treated with losartan; wherein a molar ratio based on amounts of the

losartan to E-3174 is indicative of a CYP 2C9 phenotype of said individual.

According to yet another aspect of this invention there is provided a competitive enzyme linked
5 immunosorbent assay (ELISA) kit for determining a CYP 2C9 phenotype, which comprises at least two antibodies, one specific to (s)-ibuprofen and another specific to 2-carboxyibuprofen, for detecting their molar ratio in a biological sample of an individual after consuming a
10 dose of (s)-ibuprofen wherein said molar ratio is indicative of the CYP2C9 phenotype of said individual.

According to yet another aspect of this invention there is provided a competitive enzyme linked immunosorbent assay (ELISA) kit for determining a CYP
15 2C9 phenotype, which comprises at least two antibodies, one specific to losartan and another specific to E-3174, for detecting their molar ratio in a biological sample of an individual after consuming a dose of losartan wherein said molar ratio is indicative of the
20 CYP2C9 phenotype of said individual.

According to still a further aspect of this invention, the probe substrate to be used is a dose of (s)-ibuprofen. An individual to be phenotyped consumes the probe substrate, and the individual's urine is

collected 4 hours after consumption. Urine samples are subsequently analyzed via the ELISA technology of the present invention. In particular, the urine samples are analysed for respective amounts of (s)-ibuprofen
5 and 2-carboxyibuprofen and the ratio thereof is calculated. Based on this ratio, an individual's CYP 2C9 metabolic phenotype can be characterized.

According to still a further aspect of this invention, the probe substrate to be used is a dose of
10 losartan. An individual to be phenotyped consumes the probe substrate, and the individual's urine is collected 4 hours after consumption. Urine samples are subsequently analyzed via the ELISA technology of the present invention. In particular, the urine samples
15 are analysed for respective amounts of losartan and E-3174 and the ratio thereof is calculated. Based on this ratio, an individual's CYP 2C9 metabolic phenotype can be characterized.

According to yet a further aspect of the present
20 invention there is provided derivatives of (s)-ibuprofen and 2-carboxyibuprofen and uses thereof.

According to yet a further aspect of the present invention there is provided derivatives of losartan and E-3174 and uses thereof.

The term "phenotypic determinant" is intended to mean a qualitative or quantitative indicator of an enzyme-specific capacity of an individual.

The term "individualization" as it appears
5 herein with respect to therapy is intended to mean a therapy having specificity to at least an individual's phenotype as calculated according to a predetermined formula on an individual basis.

The term "biological sample" is intended to mean
10 a sample obtained from a biological entity and includes, but is not to be limited to, any one of the following: tissue, cerebrospinal fluid, plasma, serum, saliva, blood, nasal mucosa, urine, synovial fluid, microcapillary microdialysis and breath.

15

BRIEF DESCRIPTION OF THE DRAWINGS

Fig. 1 illustrates structures of (s)-ibuprofen and 2-carboxyibuprofen;

Fig 2 illustrates structures of losartan and E-3174;

Fig. 3 illustrates (s)-ibuprofen derivatives for CYP 2C9 phenotyping by ELISA;

20 Fig. 4 illustrates 2-carboxyibuprofen derivatives for CYP 2C9 phenotyping by ELISA;

Fig 5 illustrates losartan derivatives for CYP 2C9 phenotyping by ELISA;

Fig 6 illustrates E-3174 derivatives for CYP 2C9 phenotyping by ELISA; and

5 Fig. 7 illustrates a pattern of samples to be added to a 96-well microtest plate.

DETAILED DESCRIPTION OF THE INVENTION

CYP 2C9

The CYP2C9 family of metabolic enzymes accounts
10 for approximately 8% of the metabolic enzymes in the liver. CYP 2C9 has been postulated as participating in approximately 15% of drug metabolism. Accordingly, the ability to determine an individual's capacity for CYP 2C9-specific metabolism prior to treatment with a drug
15 known to be metabolized, at least in part by the CYP 2C9 pathway would be advantageous. Furthermore, the ability to determine a CYP 2C9-specific phenotype according to the present invention will allow for the individualization of therapy with CYP 2C9-specific
20 treatments.

Polymorphism

Individuals are genetically polymorphic with respect to CYP 2C9 metabolism. Two metabolic phenotypes can be distinguished: extensive and poor metabolizers. Three genetic polymorphisms have been

5 definitively identified, one wild type (CYP2C9*1) and two mutants (CYP2C9*2 and CYP2C9*3). The CYP2C9*2 allele was found to result in 5- to 10-fold increase in expression of mRNA and have a 3-fold higher enzyme activity for metabolism of phenytoin and tolbutamide.

10 Conversely, this genotype appears to have a lower level of activity for the metabolism of S-warfarin. The CYP2C9*3 allele appears to demonstrate decreased metabolic activity against all three of these substrates.

15 CYP 2C9 metabolizes a variety of compounds including S-warfarin, phenytoin, tolbutamide, tienilic acid, and a number of nonsteroidal anti-inflammatory drugs such as diclofenac, piroxicam, tenoxicam, ibuprofen, and acetylsalicylic acid. The following

20 table (Table 1) provides a much more detailed listing of CYP 2C9 substrates.

Table 1
CYP 2C9 Substrates

Category	Subcategory	Chemical/Drug
Analgesic, antipyretic, anti-inflammatory	NSAID, Propionic acid deriv., Cyclooxygenase inhibitor	Aceclofenac
Analgesic, antipyretic, anti-inflammatory	Analgesic, Antipyretic, p-Aminophenol	Acetaminophen, Paracetamol
Analgesic, antipyretic, anti-inflammatory	Analgesic, Opioid, Diphenylheptane	Acetylmethadol, L-alpha-
Analgesic, antipyretic, anti-inflammatory	Analgesic, Opioid, Diphenylheptane	Acetylmethadol, nor-L-alpha-
Analgesic, antipyretic, anti-inflammatory	NSAID, Antiplatelet, Salicylate, Cyclooxygenase inhibitor	Acetylsalicylic acid, Aspirin
Analgesic, antipyretic, anti-inflammatory	NSAID, Pyrazolone, Cyclooxygenase inhibitor (less potent)	Aminopyrine, Amidopyrine, Aminophenazone
Analgesic, antipyretic, anti-inflammatory	NSAID, Pyrazolone, Prostaglandin synthesis inhibitor	Antipyrine, Phenazone
Analgesic, antipyretic, anti-inflammatory	NSAID, Cyclooxygenase-II inhibitor	Celecoxib
Analgesic, antipyretic, anti-inflammatory	NSAID, Phenylacetic acid deriv., Cyclooxygenase-II inhibitor	Diclofenac (used as test/marker substrate)
Analgesic, antipyretic, anti-inflammatory	NSAID, Phenylacetic acid deriv., Cyclooxygenase-II inhibitor	Diclofenac, 5-hydroxy
Analgesic, antipyretic, anti-inflammatory	NSAID, Phenylacetic acid deriv., Cyclooxygenase-II inhibitor	Diclofenac, CH(2)OH derivative
Analgesic, antipyretic, anti-inflammatory	NSAID, Cyclooxygenase-II inhibitor	Etoricoxib
Analgesic, antipyretic, anti-inflammatory	NSAID, Propionic acid deriv., Cyclooxygenase inhibitor	Flurbiprofen (S,R)-, (S)-, (R)- (used as test/marker substrate)
Analgesic, antipyretic, anti-inflammatory	NSAID, Propionic acid deriv., Cyclooxygenase inhibitor	Ibuprofen (S,R)-
Analgesic, antipyretic, anti-inflammatory	NSAID, Indole acetic acid deriv., Cyclooxygenase inhibitor	Indomethacin
Analgesic, antipyretic, anti-inflammatory	NSAID, Oxicam, Cyclooxygenase inhibitor	Lornoxicam
Analgesic, antipyretic, anti-inflammatory	NSAID, Anthranilic acid deriv., Cyclooxygenase inhibitor	Mefenamic acid
Analgesic, antipyretic, anti-inflammatory	NSAID, Oxicam, Cyclooxygenase inhibitor	Meloxicam
Analgesic, antipyretic, anti-inflammatory	Analgesic, Opioid, Diphenylheptane deriv.	Methadone, (S)- and (R)-
Analgesic, antipyretic, anti-inflammatory	NSAID, Propionic acid deriv., Cyclooxygenase inhibitor	Naproxen (S,R)-, (S)-, (R)- (used as test/marker substrate)
Analgesic, antipyretic, anti-inflammatory	Analgesic, Antipyretic, p-Aminophenol	Phenacetin
Analgesic, antipyretic, anti-inflammatory	NSAID, Pyrazolone, Cyclooxygenase inhibitor	Phenylbutazone

Analgesic, antipyretic, anti-inflammatory	NSAID, Oxicam, Cyclooxygenase inhibitor	Piroxicam (used as test/marker substrate)
Analgesic, antipyretic, anti-inflammatory	NSAID, Propionic acid deriv.	S-2-[4-(3-Methyl-2-thienyl)phenyl]propionic acid (S-MTPPA)
Analgesic, antipyretic, anti-inflammatory	NSAID, Propionic acid deriv., Cyclooxygenase inhibitor	Suprofen
Analgesic, antipyretic, anti-inflammatory	NSAID, Oxicam, Cyclooxygenase inhibitor	Tenoxicam
Antibacterial	Antileprotic	dapsone
Antibacterial	Sulphonamide	Sulfadiazine
Antibacterial	Sulphonamide	Sulfamethoxazole
Antibacterial	Sulphonamide	Sulfamethoxazole
Antibacterial	Diaminopyrimidine	Trimethoprim
Antidepressant	Tricyclic; P-Glycoprotein (P-gp) weak inhibitor	Amitriptyline
Antidepressant	Monoamine oxidase type B (MAO-B) inhibitor	Deprenyl (Selegiline)
Antidepressant	Selective serotonin reuptake inhibitor, SSRI	Fluoxetine rac., (S)-, (R)-
Antidepressant	Selective α_2 -adrenoreceptor antagonist, Piperazinoazepine	Mirtazapine
Antidepressant	Selective serotonin reuptake inhibitor, SSRI	Sertraline
Antidepressant	Serotonin and noradrenaline (norepinephrine) reuptake inhibitor, Phenylethylamine	Venlafaxine
Antidiabetic	Thiazolidinedione	Rosiglitazone
Antidiabetic	Sulphonylurea; P-Glycoprotein (P-gp) weak inhibitor	Tolbutamide (used as test/marker substrate)
Antidiabetic	Thiazolidinedione	Troglitazone
Antiepileptic	Hydantoin	Mephentoin (S)-
Antiepileptic	Barbiturate	Phenobarbital
Antiepileptic	Hydantoin	Phenytoin
Antiepileptic	Oxazolidinedione	Trimethadione (Troxidone)
Antiepileptic	Valproate	Valproic acid
Antigout	Uricosuric	Sulfinpyrazone sulfide
Antihistamine (Histamine H1- receptor antagonist)	Piperazine	Cinnarizine
Antihistamine (Histamine H1- receptor antagonist)	Piperazine	Flunarizine
Antimalarial	Naphthoquinone	58C80
Antimalarial	Artemisinin derivative	Artelinic acid
Antimalarial	Artemisinin derivative	Artelinic acid
Antimalarial	Biguanide	Proguanil

Antimuscarinic	Tertiary amine	Tolterodine
Antineoplastic	Alkylating, oxazaphosphorine	Cyclophosphamide
Antineoplastic	Alkaloid	Ellipticine
Antineoplastic	Alkylating, oxazaphosphorine	Ifosfamide
Antineoplastic	Antiestrogen, nonsteroidal	Tamoxifen
Antineoplastic	Selective retinoid X receptor modulator	Targretin (LGD1069)
Antineoplastic	Nitrosourea	Tauromustine
Antipsychotic	Dopamine D2, serotonin ₂ (5-HT ₂) and 5-HT _{1C} receptor antagonist, Dibenzodiazepine	Clozapine
Antipsychotic	Phenothiazine	Perazine
Antipsychotic	Phenothiazine; P-Glycoprotein (P-gp) inhibitor	Perphenazine
Antiviral	HIV protease inhibitor; P-Glycoprotein (P-gp) substrate/inducer	Amprenavir
Antiviral	HIV protease inhibitor; P-Glycoprotein (P-gp) substrate/inhibitor/inducer	Nelfinavir mesylate
Antiviral	HIV-1 non-nucleoside reverse transcriptase inhibitor	Nevirapine
Antiviral	Nucleoside reverse transcriptase inhibitor	Zidovudine (Azidothymidine, AZT)
Anxiolytic, sedative, hypnotic	Benzodiazepine	Desmethyldiazepam, N-
Anxiolytic, sedative, hypnotic	Benzodiazepine	Diazepam
Anxiolytic, sedative, hypnotic	Benzodiazepine	Flunitrazepam
Anxiolytic, sedative, hypnotic	Barbiturate	Hexobarbital
Anxiolytic, sedative, hypnotic	Benzodiazepine	Temazepam
Anxiolytic, sedative, hypnotic	Insomnia agent, Imidazopyridine	Zolpidem
Bronchodilators and Anti-asthma	5-Lipoxygenase inhibitor	ABT-761 and ABT-438 (metabolite, N-hydroxy)
Bronchodilators and Anti-asthma	Leucotriene D ₄ (LTD ₄) receptor selective antagonist	Zafirlukast
Cardiovascular	Thromboxane A ₂ (TXA ₂) receptor antagonist	(+)-(S)-145 and derivatives
Cardiovascular	Anticoagulant	Acenocoumarol (R / S)-
Cardiovascular	beta-Adrenoceptor blocking agent (Beta blocker)	Bufuralol
Cardiovascular	Angiotensin II receptor antagonist (sartan)	Candesartan
Cardiovascular	beta-Adrenoceptor blocking	Carvedilol (S)- and (R)-

	agent (Beta blocker); P-Glycoprotein (P-gp) inhibitor	
Cardiovascular	Anticoagulant	Dicoumarol
Cardiovascular	Calcium-channel blocker, benzothiazepine; P-Glycoprotein (P-gp) substrate/weak inhibitor	Diltiazem
Cardiovascular	Carbonic anhydrase inhibitor	Dorzolamide
Cardiovascular	Angiotensin II receptor antagonist	Irbesartan
Cardiovascular	Angiotensin II receptor antagonist (sartan); P-Glycoprotein (P-gp) substrate	Losartan
Cardiovascular	Anticoagulant	Phenprocoumon
Cardiovascular	Antiarrhythmic, Cinchona alkaloid, 4-Methanolquinoline; P-Glycoprotein (P-gp) inhibitor/substrate	Quinidine
Cardiovascular	Antiplatelet, Thromboxane A2 (TXA2) receptor antagonist	Seratrovast
Cardiovascular	Diuretic and uricosuric	Tienilic acid
Cardiovascular	Diuretic, loop	Torsemide (Torsemide)
Cardiovascular	Calcium-channel blocker, phenylalkylamine; P-Glycoprotein (P-gp) inhibitor	Verapamil, rac, (R)-, (S)-
Cardiovascular	Anticoagulant	Warfarin (S,R)-, (S)- and (R)-
Cough suppressant	Centrally acting	Dextromethorphan
Dermatological agent	Retinoic acid receptor modulator	Retinoic acid, 9-cis- (Panretin)
Dermatological agent	Retinoic acid receptor modulator	Retinoic acid, all trans- (Tretinoin)
Erectile dysfunction	cGMP-specific phosphodiesterase type 5 inhibitor	Sildenafil (Viagra)
Gastro-intestinal	5-HT3-receptor antagonist	Alosetron
Gastro-intestinal	Serotonin-5-HT4-receptor agonist, piperidiny benzamide	Cisapride
Gastro-intestinal	5-HT3-receptor antagonist	Dolasetron
Gastro-intestinal	Benzimidazole (Omeprazole deriv.)	H 259/31
Gastro-intestinal	Proton pump inhibitor, benzimidazole	Lansoprazole
Gastro-intestinal	Proton pump inhibitor, pyridinylsulfinylbenzimidazole; P-Glycoprotein (P-gp) inhibitor	Omeprazole (S)- (Esomeprazole)
Gastro-intestinal	5-HT3-receptor antagonist	Ondansetron
Gastro-intestinal	Natural compound, Antiemetic, Marijuana (Cannabis) const.	Tetrahydrocannabinol THC, delta1- (delta9-)
Gastro-intestinal	5-HT3-receptor antagonist	Tropisetron
Gastro-intestinal	5-Lipoxygenase inhibitor	Zileuton
Gastro-intestinal	5-Lipoxygenase inhibitor	Zileuton, N-dehydroxy

		metabolite
General anesthetic	Halogenated	Halothane
General anesthetic	NMDA receptor antagonist, phencyclidine deriv.	Ketamine (R)-, (S)-
General anesthetic	Di-isopropylphenol	Propofol
General anesthetic	Thiobarbiturate	Thiamylal (S)- and (R)-
Lipid regulating	HMG-CoA reductase inhibitor (Statin); P-Glycoprotein (P-gp) substrate	Fluvastatin
Lipid regulating	HMG-CoA reductase inhibitor	NK-104
Local anesthetic	Amide type	Lidocaine (Lignocaine)
Other chemical	Polycyclic aromatic hydrocarbon (PAH)	(+)- and (-)-7,8-Dihydroxy-7,8-dihydro-benzo[a]pyrene, (+)- and (-)-B[a]P-7,8-diol
Other chemical	Polycyclic aromatic hydrocarbon (PAH)	(+)-(11S,12S)- and (-)-(11R,12R)-Dihydroxydibenzo[a,l]pyrene (DB[a,l]P-11,12-diol)
Other chemical	Polycyclic aromatic hydrocarbon (PAH)	1,2-Dihydroxy-1,2-dihydro-5,6-dimethylchrysene (5,6-Dimethylchrysene-1,2-diol)
Other chemical	Polycyclic aromatic hydrocarbon (PAH)	11,12-Dihydroxy-11,12-dihydrobenzo[g]chrysene (Benzo[g]chrysene-11,12-diol, B[g]C-11,12-diol)
Other chemical	Heterocyclic amine	2-Amino-1-methyl-6-phenylimidazo[4,5-b]pyridine, PhIP
Other chemical	Heterocyclic, aromatic amine	2-Amino-3,4-dimethylimidazo[4,5-f]quinoline, MeIQ
Other chemical	Heterocyclic, aromatic amine	2-Amino-3-methylimidazo[4,5-f]quinoline, IQ
Other chemical	Aromatic amine, Arylamine	2-Amino-6-methyldipyrido[1,2-a:3,2'-d]-imidazole, Glu-P-1
Other chemical	Aromatic amine, Arylamine	2-Aminoanthracene, 2-AA
Other chemical	Aromatic amine, Arylamine	2-Aminofluorene, 2-AF
Other chemical	2-Aroylthiophene	2-Aroylthiophenes (bearing negative charge)
Other chemical	Polycyclic aromatic hydrocarbon (PAH)	3,4-Dihydroxy-3,4-dihydrobenzo[c]phenanthrene (Benzo[c]phenanthrene-3,4-diol, B[c]P-3,4-diol)
Other chemical	Heterocyclic, aromatic amine	3-Amino-1,4-dimethyl-5H-pyrido[4,3-b]indole (Trp-P-1)
Other chemical	Heterocyclic, aromatic amine	3-Amino-1-methyl-5H-pyrido[4,3-b]indole (Trp-P-2)
Other chemical	Alkyloxycoumarin	3-Cyano-7-ethoxycoumarin
Other chemical	Alkyloxyfluorescein	3-O-methylfluorescein (used

		as test/marker substrate)
Other chemical	Polycyclic aromatic hydrocarbon (PAH)	7,12-Dimethylbenz[a]anthracene (7,12-DMBA)
Other chemical	Polycyclic aromatic hydrocarbon (PAH)	7,12-Dimethylbenz[a]anthracene-3,4-diol (7,12-DMBA-3,4-diol)
Other chemical	Polycyclic aromatic hydrocarbon (PAH)	7,8-Dihydroxy-7,8-dihydrobenzo[a]pyrene, B[a]P-7,8-diol
Other chemical	Alkyloxycoumarin	7-Benzoyloxy-4-trifluoromethylcoumarin, BFC
Other chemical	Alkyloxycoumarin	7-Ethoxy-4-trifluoromethylcoumarin
Other chemical	Alkyloxycoumarin	7-Ethoxycoumarin
Other chemical	Polycyclic aromatic hydrocarbon (PAH)	Benz[a]anthracene (1,2-Benzanthracene)
Other chemical	Polycyclic aromatic hydrocarbon (PAH)	Benz[a]anthracene-3,4-diol
Other chemical	Polycyclic aromatic hydrocarbon (PAH)	Benzo[a]pyrene, B[a]P
Other chemical	Polycyclic aromatic hydrocarbon (PAH)	Benzo[b]fluoranthene-9,10-diol (B[b]F-9,10-diol)
Other chemical	Endocrine disruptor, estrogen activity	Bisphenol A
Other chemical	Unclassified	Butadiene monoxide (Epoxybutene)
Other chemical	Polycyclic aromatic hydrocarbon (PAH)	Chrysene-1,2-diol
Other chemical	Polycyclic aromatic hydrocarbon (PAH)	Dibenzo[a,h]anthracene
Other chemical	Polycyclic aromatic hydrocarbon (PAH)	Dibenzo[a,l]pyrene (DB[a,l]P)
Other chemical	Polycyclic aromatic hydrocarbon (PAH)	Dibenzo[a]pyrene
Other chemical	Alkyloxyfluorescein	Dibenzylfluorescein, DBF (suggested as test/marker substrate)
Other chemical	Alkyloxyfluorescein	Diethoxy (-5/-6) chloromethyl fluorescein (DECMF)
Other chemical	Polycyclic aromatic hydrocarbon (PAH)	Naphthalene
Other chemical	Nonionic phenolic detergent	Triton N-101
Pesticide	Insecticide, Chlorinated	Methoxychlor
Physiological compound	Steroid	5alpha-Androstane-3alpha, 17beta-diol
Physiological compound	Fatty acid	Arachidonic acid
Physiological	Fatty acid	Linoleic acid

compound		
Physiological compound	Hormone, Methoxytryptamine	Melatonin
Sex hormone	Progestagen	Desogestrel
Sex hormone	Estrogen	Estradiol, 17beta-
Sex hormone	Estrogen	Estradiol, 3-methyl ether
Sex hormone	Estrogen	Estrone
Sex hormone	Estrogen, synthetic, contraceptive	Mestranol
Sex hormone	Progestagen; P-Glycoprotein (P-gp) inhibitor	Progesterone
Sex hormone	Androgen and anabolic	Testosterone
Supplementary drugs and other substances	Natural compound, Monoterpene cyclic ether, Eucalyptus polybractea const.	1,8-Cineole (Eucalyptol)
Supplementary drugs and other substances	Schizandrin C deriv., used as hepatoprotective	DDB
Supplementary drugs and other substances	Natural compound, Garlic oil component, Organosulfur	Diallyl disulfide (DADS)
Supplementary drugs and other substances	Natural compound, Alkaloid	Nicotine
Supplementary drugs and other substances	Natural compound, Mycotoxin	Ochratoxin A
Supplementary drugs and other substances	Antialcoholic	S-methyl N,N,-diethylthiolcarbamate (DETC-ME)
Supplementary drugs and other substances	Natural compound, Marijuana (Cannabis) const.	Tetrahydrocannabinol THC, 7alpha-hydroxy-delta8-
Xanthine	Bronchodilator	Theophylline
Xanthine, Food component	CNS stimulant; P-Glycoprotein (P-gp) weak inhibitor	Caffeine

Induction and Inhibition

CYP 2C9 is inhibited by fluconazole, metronidazole, miconazole, ketoconazole, itaconazole, ritonavir, clopidrogel, amiodarone, fluvoxamine, sulfamthoxazole, fluvastatin and fluoxetine. It is

5 induced by rifampin and rifabutin. The ability to quickly and easily determine an individual's CYP 2C9-specific phenotype allows a physician to determine the

phenotypic status of an individual and make a corresponding determination about the type and extent of treatment most suitable at a given time. The present invention provides a reliable method of
5 identifying a suitable drug compatible with an individual's phenotype, as well as a method of individualizing therapy with a specific drug(s) with respect to dosage, duration etc. based thereon.

In accordance with an embodiment of the present
10 invention there is provided a phenotypic determinant specific for CYP 2C9 metabolism. This phenotypic determinant provides an indication of an individual's CYP 2C9 phenotype. Furthermore, the phenotypic determinant may be used to provide a drug response
15 profile for the individual specific to drug(s) known to be metabolized by the CYP 2C9 pathway.

Inter Ethnic Differences

The CYP 2C9 genotypes demonstrate marked inter-ethnic variability. The CYP2C9*2 is absent from
20 Chinese, Taiwanese and present in only 1% of African American populations, but accounts for 19.2% of the British population and 8% of Caucasians. CYP2C9*3 is more rare and is present in 6% of Caucasian, 2% of

Chinese, 2.6% of Taiwanese and 0.5% of African-American populations.

It is reasonable that, in drug metabolism studies, each ethnic group can be studied separately
5 for evidence of polymorphism and its antimode should not be extrapolated from one ethnic population to another. Furthermore, this inter-ethnic variability provides a clear indication that efforts to individualization treatments should be made to overcome
10 the risks and inefficiencies currently experienced with standardized dosing.

S-warfarin

As an example, the benefit of CYP 2C9 metabolic phenotyping in drug dosing is evident in the case of S-warfarin.
15 S-warfarin is an anticoagulant drug. Studies have demonstrated that the presence of either CYP2C9*2 or CYP2C9*3 haplotypes-mutants results in a decrease in the dose necessary to acquire target anticoagulation intensity. In addition, these
20 individuals also suffered from an increased incidence of bleeding complications. Therefore, the CYP 2C9 gene variants modulate the anticoagulant effect of the dose of warfarin prescribed. Clearly, the ability to

readily determine the presence of such mutant alleles prior to treatment would prove beneficial as a compatible dosage of S-warfarin could then be determined. Thus alleviating or eliminating the
5 occurrence of adverse side effects.

For these reasons, the utility of a reliable test for CYP 2C9 is evident. In particular, an accurate and convenient clinical assay would allow physicians to quickly identify safe and effective
10 treatment regimes for individuals on an individual basis. In addition, the present invention provides a means to determine the efficiency of an individual's CYP 2C9 metabolism before prescribing a standard treatment. In doing so, a standard treatment may then
15 be tailored to provide an individualized treatment that will correspond with an individual's CYP 2C9 phenotype.

Direct Phenotypic Determinants of CYP 2C9

Different substrates (or probe substrates) such
20 as ibuprofen, losartan, tolbutamide, lurbiprofen, diclofenac, phenytoin & warfarin can be used to determine a CYP 2C9 phenotype according to the present invention. (s)-ibuprofen is exemplified as a probe

substrate, without limitation, in accordance the present invention.

According to one embodiment of the present invention, the ratio of (s)-ibuprofen and its
5 carboxylated metabolite, 2-carboxyibuprofen in a urine sample may be used to provide a phenotypic determinant corresponding to an individual's CYP 2C9 phenotype. This metabolite is used as a quantitative marker in the determination of a CYP 2C9 phenotype on the basis of
10 the use of the preferred probe substrate (s)-ibuprofen. The structures of (s)-ibuprofen and its metabolite 2-carboxyibuprofen are illustrated in Fig. 1. However, it is fully contemplated that the present invention is not limited in any respect thereto. In fact, due to
15 the nature of the substrate specific alterations caused by the individual CYP 2C9 mutations, multiple probe substrates may be employed for a phenotypic determination of CYP 2C9.

The molar ratio of (s)-ibuprofen and its 2-
20 carboxyibuprofen metabolite, used to determine the CYP 2C9 phenotype of the individual, is as follows:

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(s)-ibuprofen
2-carboxyibuprofen

According to another embodiment of the present invention, the ratio of losartan and its metabolite E-3174 in a urine sample may be used to provide a phenotypic determinant corresponding to an individual's CYP 2C9 phenotype. This metabolite is used as a quantitative marker in the determination of a CYP 2C9 phenotype on the basis of the use of the preferred probe substrate losartan. The structures of losartan and its metabolite E-3174 are illustrated in Fig. 2. However, it is fully contemplated that the present invention is not limited in any respect thereto. In fact, due to the nature of the substrate specific alterations caused by the individual CYP 2C9 mutations, multiple probe substrates may be employed for a phenotypic determination of CYP 2C9.

The molar ratio of losartan and its metabolite E-3174, used to determine the CYP 2C9 phenotype of the individual, is as follows:

Losartan
E-3174

Enzyme linked immunosorbent assays (ELISA) have been successfully applied in the determination of low

amounts of drugs and other antigenic compounds in plasma and urine samples and are simple to carry out. An ELISA for N-acetyltransferase-2 (NAT2) phenotyping using caffeine as a probe substrate has also been
5 developed and validated (Wong, P., Leyland-Jones, B., and Wainer, I.W. (1995) J. Pharm. Biomed. Anal. **13**: 1079-1086); (Leyland-Jones *et al.* (1999) Amer. Assoc. Cancer Res. **40**: Abstract 356). The ELISA for NAT2 phenotyping is simpler to carry out than the HPLC and
10 CE.

In developing the antigen enzyme linked immuno-sorbent assay (ELISA) of the present invention, antibodies to (s)-ibuprofen and 2-carboxyibuprofen have been developed to measure the molar ratio of these
15 compounds in urine samples collected from an individual after (s)-ibuprofen consumption. The antibodies of the present invention can be polyclonal or monoclonal antibodies raised against derivatives of (s)-ibuprofen and 2-carboxyibuprofen, as exemplified in Figs. 3 and
20 4, respectively. Based on the development of these derivatives and subsequently derived antibodies, the ability to determine the molar ratio of (s)-ibuprofen and 2-carboxyibuprofen, in accordance with the present invention, was achieved.

In accordance with an embodiment of the present invention the antibodies of the present invention can be polyclonal or monoclonal antibodies raised against derivatives of losartan and E-3174, as exemplified in
5 Figs. 5 and 6, respectively.

In accordance with an embodiment of the present invention, the ratio of (s)-ibuprofen and 2-carboxyibuprofen in a urine sample may be used to provide a determination of an individual's CYP 2C9
10 phenotype. These compounds are used as quantitative markers in the determination of a CYP 2C9 phenotype on the basis of the use of the preferred probe substrate (s)-ibuprofen. However, it is fully contemplated that the present invention is not limited in any respect
15 thereto.

In accordance with an embodiment of the present invention, the ratio of losartan and E-3174 in a urine sample may be used to provide a determination of an individual's CYP 2C9 phenotype. These compounds are
20 used as quantitative markers in the determination of a CYP 2C9 phenotype on the basis of the use of the preferred probe substrate losartan. However, it is fully contemplated that the present invention is not limited in any respect thereto.

In accordance with another embodiment of the present invention, a competitive antigen ELISA is provided for determining CYP 2C9 phenotyping using (s)-ibuprofen as the probe substrate. The assay is
5 sensitive, rapid and can be readily carried out on a routine basis by a technician with a minimum of training in a clinical laboratory.

The present invention will be more readily understood by referring to the following Materials and
10 Methods and Examples which are given to illustrate the invention rather than to limit its scope.

MATERIALS AND METHODS

Materials

Horse radish peroxidase is purchased from Boehringer Mannheim (Montreal, Que., Canada); ELISA
15 plates (96-well Easy WashTM modified flat bottom, high binding); Corning glass wares, (Corning, NY, USA) and Falcon 96-well microtest tissue culture plate, no. 3072 (Beckton Dickinson Labware, Franklin, NJ, USA) are purchased from Fisher (Montreal, Que., Canada);
20 alkaline phosphatase conjugated to goat anti-rabbit IgGs, Keyhole limpet hemocyanin (KLH) is from Pierce Chemical Co. (Rockford, IL, USA); acetic anhydride,

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acetonitrile HPLC grade, benzylurea, bovine serum albumin (Cat. No. A-3803), N-bromosuccinimide, 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride solution (EDAC), diethanolamine, Freund's
5 adjuvant (complete and incomplete), glutaraldehyde (50% v/v), p-nitrophenolphosphate disodium salt, palladium, 10 wt.% (dry basis) on activated carbon, o-phenylenediamine hydrochloride, polyoxyethylene sorbitan monolaurate (TweenTM 20), porcine skin gelatin,
10 protein A-Sepharose 4B, SephadexTM G25 fine, sodium hydride, tributylamine, TweenTM 20, are purchased from Sigma-Aldrich (St-Louis, MO, USA); Silica gel particle size 0.040-0.063 mm (230-400 mesh) ASTM Emerck Darmstadt, Germany is purchased from VWR (Montreal,
15 Que., Canada). Dioxane is dried by refluxing over calcium hydride for 4 hours and distilled before use. Other reagents are ACS grade.

Synthesis of Derivatives of (s)-ibuprofen and 2-carboxyibuprofen

The (s)-ibuprofen and 2-carboxyibuprofen
20 derivatives may include, without limitation those illustrated in Figs. 3 and 4.

Conjugation of haptens to bovine serum albumin (BSA)
and keyhole limpet hemocyanin

(s)-Ibuprofen-BSA and 2-carboxyibuprofen-BSA
conjugates are prepared by a procedure similar to that
of Rojo et al. (Rojo et al. (1986) J Immunol. **137**:
904-910). In a 25 mL erlenmeyer flask 15 mg of BSA is
5 dissolved in 6 mL of a (s)-ibuprofen derivative (or 2-
carboxyibuprofen derivative) solution (1.25 μ moles/mL
of water) followed by the addition of 1.43 mL of an
EDAC solution (10 mg/mL of water). The solution is
stirred overnight at room temperature and dialyzed
10 against 500 mL water at room temperature for 48 hours
with two changes per day of the water. The conjugates
are stored as 0.5 mL-aliquots at -20°C. In addition,
the conjugates may be prepared by the method of Peskar
et al. (Peskar (1972) Eur. J. Biochem. **26**: 191-195).
15 In a 5 mL round bottom flask 7.5 mg of (s)-ibuprofen
derivative (or 2-carboxyibuprofen derivative)
(0.03 mmole) is placed and is dissolved with 1 mL of a
0.1M Na₂HPO₄-NaH₂PO₄ buffer, pH 7.0. A volume of 500 μ L
of a 0.021 M glutaraldehyde solution (42.5 μ L 50%
20 glutaraldehyde (v/v) per 10 mL of water) is added to
the stirred solution. After stirring for 2 hours,

100 μ L of a 1M lysine solution in 0.1M Na_2HPO_4 - NaH_2PO_4 buffer, pH 7.0 is added. The solution is stirred for one hour and dialyzed against 250 mL of a 150 mM NaCl, 5 mM Na_2HPO_4 - NaH_2PO_4 buffer, pH 7.0 for 48 hours with 2-3
5 changes per day of the buffer. Solution BSA conjugates are stored as 0.5 mL aliquots at -20°C .

(s)-Ibuprofen-KLH and 2-carboxyibuprofen-KLH conjugates are prepared as follows. First, 20 mg of lyophilized powder of KLH is dissolved with 2 mL of a
10 0.9 M NaCl solution and dialyzed against 100 mL of water for 10 hours with 2 changes of the water. To 1.1 mL KLH solution (~ 10 mg/mL) in a 25 mL erlenmeyer flask, 0.8 mL of the (s)-ibuprofen derivative (or 2-carboxyibuprofen derivative) ($2.5 \mu\text{mol/mL}$ in
15 0.9 M NaCl). 2 mL of an EDAC solution (10 mg/mL in 0.9 M NaCl), and 1.8 mL 0.9 M NaCl solution are successively added to the derivative solution. The solution is stirred overnight (20 hours) at room temperature. The solution is dialyzed against 250 mL
20 of a 0.9 M NaCl solution for 48 hours with 2-3 changes of the solution per day. (s)-ibuprofen-KLH and 2-carboxyibuprofen-KLH solutions are stored as 0.5 mL aliquots at -20°C . In addition, the conjugates may be prepared according to a method similar to that of

Peskar *et al.* (Peskar (1972) *Eur. J. Biochem.* **26**: 191-195). First, 20 mg of lyophilized powder of KLH is dissolved with 2 mL of a 0.9 M NaCl solution and dialyzed against 100 mL of water for 10 hours with 2
5 changes of the water. Approximately 0.03 mmole of (s)-ibuprofen or 2-carboxyibuprofen is placed in a 5 mL round bottom flask and is dissolved with 1 mL of the KLH solution. A volume of 500 μ L of a 0.021 M glutaraldehyde solution (42.5 μ L 50% glutaraldehyde
10 (v/v) per 10 mL of water) is added dropwise to the stirred solution. After stirring for 2 hours, 100 μ L of a 1M lysine solution in 0.1M Na₂HPO₄-NaH₂PO₄ buffer, pH 7.0 is added. The solution is stirred for one hour and dialyzed against 250 mL of a 0.9M NaCl, 5 mM
15 Na₂HPO₄-NaH₂PO₄ buffer, pH 7.0 for 48 hours with 2-3 changes per day of the buffer. Solutions of BSA conjugates are stored as 0.5 mL aliquots at -20°C.

Protein Determination

Protein determination was performed according to
20 the method of Lowry *et al.* as described in Lowry, O.H. *et al.* (1951) *J. Biol. Chem.*, 193: 265-275, which is herein incorporated by reference.

Solutions

Solution A: 2g Na_2CO_3 is dissolved in 50 mL water, 10 mL of 10% SDS and 10 mL 1N NaOH; bring to 100 mL volume with water. Freshly prepared.

Solution B: 1% NaK Tartrate

Solution C: 1% $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$

Solution D: 1N phenol (freshly prepared): 3 mL Folin & Ciaocalteu's phenol reagent (2.0 N) and 3 mL water.

Solution E: 98 mL Solution A, 1 mL Solution B, 1 mL Solution C. Freshly prepared.

BSA: 1 mg/mL. 0.10 g bovine serum albumin (fraction vol.)/100 mL water.

ASSAY

Standard curve	Tube # (13 × 100mm)						
Solution	1	2	3	4	5	6	7
BSA (μl)	0	10	15	20	30	40	50
Water (μl)	200	190	185	180	170	160	150
Solution E (mL)	2.0	2.0	2.0	2.0	2.0	2.0	2.0

The solutions are vortexed and left for 10 min at room temperature.

Solution D (μl)	200	200	200	200	200	200	200
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The solutions are vortexed and left at room temperature for 1 hour.

The absorbance is read at 750 nm using water as the blank.

UNKNOWN

<u>Solution D.F.^a (in triplicate)</u>	<u>Tube # (13 × 100 mm)</u>		
Unknown (μl)	x	x	x
Water (μl)	y	y	y
(x + y = 200 μl)			
Solution F (mL)	2.0	2.0	2.0

The solutions are vortexed and left for 10 min at room temperature.

Solution D (μl)	200	200	200
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The solutions are vortexed and left at room temperature for 1 hour.

The absorbance is read at 750 nm using water as the blank.

The protein concentration is calculated using the standard curve and taking in to account the D.F. (dilution factor) of the unknown.

a: D.F. (dilution factor): has to be such that the absorbance of the unknown at 750 nm is within the range of absorbance of the standards.

Methods to Determine the Amounts of Moles of (s)-ibuprofen or 2-carboxyibuprofen Incorporated per mg of KLH

This method gives an approximate estimate. It is useful because it allows the determination of whether the coupling proceeded as expected.

A) Solutions

- 10% sodium dodecyl sulfate (SDS) solution
- 1% SDS solution
- 0.5 or 1 mg/mL of (s)-ibuprofen-KLH (or 2-carboxyibuprofen-KLH) in a 1% SDS solution (1 mL)
- 0.5 or 1 mg/mL KLH in a 1% SDS solution

B) Procedure

- The absorbance of the (s)-ibuprofen-KLH conjugate (or 2-carboxyibuprofen-KLH) is measured at the wavelength of absorption maximum of (s)-ibuprofen, with a 1% SDS solution as the blank.
- The absorbance of the KLH solution is measured at the wavelength of absorption maximum of (s)-ibuprofen, with a 1% SDS solution as the blank.
- The amount of mole of (s)-ibuprofen incorporated per mg of KLH is calculated with the following formula:

$$y = \frac{A_{\lambda_{\max}}(\text{ibuprofen-KLH}) - A_{\lambda_{\max}}(\text{KLH})}{\epsilon_{\lambda_{\max}}(\text{ibuprofen}) \times [\text{KLH}]}$$

where:

y is the amount of mole of (s)-ibuprofen/mg of KLH;

$\epsilon_{\lambda_{\max}}$ ((s)-ibuprofen) is the molar extinction coefficient of (s)-ibuprofen at the wavelength of absorption maximum.

Coupling of Haptens to Horse Radish Peroxidase

The (s)-ibuprofen and 2-carboxyibuprofen derivatives (after succinylation with succinic anhydride) are conjugated to horse radish peroxidase (HRP) by the following procedure. In a 5 mL round
5 bottom flask are placed 0.12 mmol of the derivative. Then, 500 μ L of dioxane freshly dried over calcium chloride is added. The suspension is stirred and cooled at 10°C in a water bath using crushed ice. Then, 31 μ L isobutylchloroformate (0.24 mmol) (recently
10 opened or purchased) and 114 μ L tributylamine (0.47 mmol) are added. The suspension is stirred for 30 min at 10°C. While stirring, 13 mg of horse radish peroxidase (HRP) is dissolved in 2 mL of water and the solution is cooled at 4°C on crushed ice. After the
15 30 min of stirring, 100 μ L of a 1N NaOH solution (freshly prepared) at 4°C is added to the HRP solution and the alkaline HRP solution is poured at once in the 5 mL flask. The suspension is stirred for 4 hours at 10-12°C. The free derivative is separated from the HRP

conjugate by filtration on a Sephadex G-25TM fine column (1.6 × 30 cm) equilibrated and eluted with 0.1 M sodium phosphate buffer, pH 7.0. The fractions of 1.0-1.2 mL are collected manually or with a fraction collector.

5 During elution two bands may be observed: the HRP conjugate and a light yellow band behind the HRP conjugate. The HRP conjugate band is eluted between fractions 11-16. The fractions containing the HRP conjugate are pooled in a 15 mL tissue culture with a

10 screw cap. The HRP conjugate concentration is determined at 403 nm after diluting an aliquot (usually 50 µL + 650 µL of buffer).

$$[\text{HRP conjugate}] \text{ (mg/mL)} = A_{403} \times 0.4 \times \text{D.F.}$$

After the reaction is complete, 5 µL of a 4% thiomersal solution is added per mL of (s)-ibuprofen-

15 HRP or 2-carboxyibuprofen -HRP conjugate solution. The conjugates are stored at 4°C.

Antibody Production

Four mature females New Zealand white rabbits (Charles River Canada, St-Constant, Que., Canada) are

20 used for antibody production. An isotonic saline solution (0.6 mL) containing 240 µg of KLH conjugated

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antigen is emulsified with 0.6 mL of a complete Freund's adjuvant. Then, 0.5 mL of the emulsion (100 µg of antigen) is injected per rabbit intramuscularly or subcutaneously. Rabbits are subsequently boosted at intervals of three weeks with 50 µg of antigen emulsified in incomplete Freund's adjuvant. Blood is collected without anticoagulant in a vacutainer tube by venipuncture of the ear 10-14 days after boosting and kept at 4°C. After clotting, centrifugation at 4°C, sodium azide is added to the antisera to a final concentration of 0.001% (1 µL of a 1% sodium azide solution per mL of antisera). Antisera is stored as 0.5 mL aliquots at -20°C.

Antiserum Titers

The wells of a microtiter plate are coated with 10 µg mL⁻¹ of bovine serum albumin-(s)-ibuprofen(or R-mephenytoin) conjugate in 100 mM sodium carbonate buffer, pH 9.6) overnight at 4°C (150 µL/well). The wells are then washed three times with TPBS (phosphate buffered saline containing 0.05% TweenTM 20) using a Nunc Immuno Wash 12 autoclavable. Unoccupied sites are blocked by an incubation with 150 µL/well of TPBS

containing 0.05% porcine gelatin for 2 h at room temperature. The wells are washed three times with TPBS and 150 μ L of antiserum diluted in TPBS is added. After 2 h at room temperature, the wells are washed
5 three times with TPBS, and 100 μ L of goat anti-rabbit IgGs-alkaline phosphatase conjugate diluted in PBS containing 1% BSA are added. After 1 h at room temperature, the wells are washed three times with TPBS and three times with water. To the wells are added
10 150 μ L of a solution containing $MgCl_2$ (0.5 mM) and p-nitrophenol phosphate (3.85 mM) in diethanolamine buffer (10 mM, pH 9.8). After 30 min at room temperature, the absorbency is read at 405 nm with a microplate reader. The antibody titer is defined as
15 the dilution required to change the absorbance by one unit (1 au).

Isolation of IgG Antibodies

Rabbit IgG antibodies against KLH conjugates are purified by affinity chromatography on a Protein
20 A-Sepharose 4B column as follows. A 0.9 x 15 cm Pharmacia chromatographic column is packed with Protein A-Sepharose 4B suspension to a volume of 1 mL. The

column is washed generously with a 0.01 M Na_2HPO_4 - NaH_2PO_4 buffer, pH 8.0 containing 0.15 M NaCl (PBS) and then washed with 3-4 mL of a 0.1 M trisodium citrate buffer, pH 3.0. The column is then washed generously with PBS.

5 Then, 1 mL of rabbit antiserum is diluted with 1 mL PBS, and the resulting solution is slowly applied to the column. The column is washed with 15 mL PBS and eluted with a 0.1 M trisodium citrate buffer, pH 3.0. Three fractions of 2.2 mL are collected in 15 mL

10 graduated tubes containing 0.8 mL of 1 M Tris-HCl buffer, pH 8.5. The purified rabbit IgG antibodies are stored at 4°C in the presence of 0.01% sodium azide.

Antibody Specificity

To ensure accuracy in the ELISA measurement of

15 CYP 2C9 phenotyping, the antibodies must have specificity for their individual molecules, with little or no recognition of other derivatives. To ensure their selectivity an ELISA is performed with standard solutions of (s)-ibuprofen metabolites and other

20 structurally similar compounds.

RESULTS

Positive creation of antibodies against (s)-ibuprofen and 2-carboxyibuprofen can be seen by antibody titers of 30,000-100,000 as determined by the ELISA, strong precipitation lines after double
5 immunodiffusion in agar plates of antisera and derivatives conjugated to rabbit serum albumin, and low cross-reactivity with other mephenytoin derivatives. These results constitute positive conditions for the development of a competitive antigen ELISA according to
10 the methods described in the above section entitled Materials and Methods.

EXAMPLE I**A Competitive Antigen ELISA for CYP 2C9 Phenotyping**

Buffers and water without additives are filtered through 0.45 μ M millipore filters and kept for one week, except the substrate buffer which is freshly
15 prepared. BSA, antibodies, TweenTM 20 and horse radish peroxidase are added to buffers and water just prior to use.

Preparation of Urine Samples

Urine samples are usually collected four hours after ingestion of (s)-ibuprofen and stored at -20°C as 1-mL aliquots in 1.5 mL microtubes. For the ELISA, the urine samples are diluted with isotonic sodium phosphate buffer, pH 7.5 (310 mosM) to give concentrations of (s)-ibuprofen or 2-carboxyibuprofen no higher than 3×10^{-6} M in the microtiter plate wells. Just prior to the ELISA, samples are mixed in a 1:1 ratio (e.g. 100 μL :100 μL) with either the (s)-ibuprofen-HRP or the 2-carboxyibuprofen-HRP conjugate (12 mg mL^{-1}).

Standard Solutions of 2-carboxyibuprofen or (s)-ibuprofen for ELISA

A 100 mL stock solution of (s)-ibuprofen or 2-carboxyibuprofen at concentrations of 6.00×10^{-4} M is prepared in the 310 mosM sodium phosphate buffer, pH 7.5 (IPB) in a 100 mL volumetric flask. The solution is stirred to insure complete solubilization.

The stock solutions are stored as 1 mL aliquots at -20°C . On the day of the ELISA, one aliquot is thawed and warmed up at room temperature. The

following standard solutions (Table 2) of the above compounds are prepared:

Table 2
Standard Solutions

5

Standard #	[Compound]		Composition
1	6.00×10^{-4}	M	<i>Stock Solution</i>
2	2.00×10^{-4}	M	200 μ L S1 + 400 μ OL IPB
3	1.12×10^{-4}	M	200 μ L S1 + 868 μ L IPB
4	6.00×10^{-5}	M	100 μ L S1 + 900 μ L IPB
5	3.56×10^{-5}	M	60 μ L S1 + 951 μ L IPB
6	2.00×10^{-5}	M	100 μ L S2 + 900 μ L IPB
7	1.12×10^{-5}	M	100 μ L S3 + 900 μ L IPB
8	6.00×10^{-6}	M	100 μ L S4 + 900 μ L IPB
9	3.56×10^{-6}	M	100 μ L S5 + 900 μ L IPB
10	2.00×10^{-6}	M	100 μ L S6 + 900 μ L IPB
11	1.12×10^{-6}	M	100 μ L S7 + 900 μ L IPB
12	6.00×10^{-7}	M	100 μ L S8 + 900 μ L IPB
13	3.56×10^{-7}	M	100 μ L S9 + 900 μ L IPB
14	2.00×10^{-7}	M	100 μ L S10 + 900 μ L IPB
15	1.12×10^{-7}	M	100 μ L S11 + 900 μ L IPB
16	6.00×10^{-8}	M	100 μ L S12 + 900 μ L IPB
17	3.56×10^{-8}	M	100 μ L S13 + 900 μ L IPB
18	2.00×10^{-8}	M	100 μ L S14 + 900 μ L IPB
19	2.00×10^{-9}	M	100 μ L S15 + 900 μ L IPB
20	2.00×10^{-10}	M	100 μ L S15 + 900 μ L IPB
21	2.00×10^{-11}	M	100 μ L S15 + 900 μ L IPB
22	2.00×10^{-12}	M	100 μ L S15 + 900 μ L IPB
23	2.00×10^{-13}	M	100 μ L S15 + 900 μ L IPB

ELISA Conditions

Wells of the ELISA plate are washed with a Nunc-Immuno wash 12 washer. Then, 16 mL of a solution of $6.6 \mu\text{g ml}^{-1}$ of isolated IgG antibodies is prepared in a 100 mM sodium carbonate buffer, pH 9.6, and 150 μL of this solution is pipetted in each well of a microtiter plate using a eight channel pipet (Brinkmann TransferpetteTM-8 50-200 μL) and 200 μL Flex tips from Brinkmann). After coating the wells with antibodies at 4°C for 20 h, the wells are washed 3 times with the isotonic sodium phosphate buffer containing 0.05% TweenTM 20 (IPBT) and properly drained by inverting the plate and absorbing the liquid on piece of paper towel. Next, 30 mL of a solution of a IPBT solution containing 1% BSA is prepared and 150 μL of this solution is pipetted in each well using a eight channel pipet (Brinkmann TransferpetteTM-8 50-200 μL) and 200 μL yellow tips (Sarstedt yellow tips for P200 Gilson Pipetman). After 3 h at room temperature, the wells were washed 3 times with IPBT solution and drained. Then, 400 μL of sample or standard for determination of 2-carboxyibuprofen or (s)-ibuprofen are prepared (as described in previous sections) in 1.5 mL microtubes

using Sarstedt yellow tips and a P200 Gilson Pipetman. Each sample/standard (200 μ L) is pipetted in duplicate in a Falcon 96 well microtest tissue culture plate according to the pattern shown in Fig. 7, using
5 Sarstedt yellow tips and a P200 Gilson Pipetman. Using an eight channel pipet (Brinkmann TransferpetteTM-8 50-200 μ L) and changing the tips of the eight channel pipet (200 μ L Flex tips from Brinkmann) at each row, 150 μ L of sample/standard are transferred in the
10 corresponding wells of a 96 well ELISA microtiter plate coated with antibodies. After the addition of the samples and standards, the microtiter plates are covered and left standing at room temperature for 2 h. While the plate is left standing the substrate buffer
15 without the hydrogen peroxide and o-phenylenediamine hydrochloride is prepared (25 mM citric acid and 50 mM sodium phosphate dibasic buffer, pH 5.0). The microtiter plate is washed 3 times with the IPBT solution and 3 times with a 0.05% TweenTM 20 solution
20 and drained. Next, 50 μ L of hydrogen peroxide and 40 mg of o-phenylenediamine are added to the substrate buffer. One hundred and fifty microliters (150 μ L) of the substrate buffer solution is then added to each

well using an eight channel pipet (Brinkmann TransferpetteTM-8 50-200 μ L) and 200 μ L Flex tips (Brinkmann). The microtiter plate is covered and shaken for 25-30 min at room temperature and the
5 enzymatic reaction is stopped by adding 50 μ L/well of a 2.5 M HCl solution using an eight channel pipet (Brinkmann TransferpetteTM-8 50-200 μ L) and 200 μ L Flex tips (Brinkmann). After gently shaking for 3 min, the absorbance is read at 490 nm with a microplate reader.

EXAMPLE II

Determination of (s)-ibuprofen and 2-carboxyibuprofen
in Urine Samples with the ELISA kit

The contents of an ELISA kit for determining CYP
2C9 phenotype are exemplified in Table 3.

Table 3
Content of the ELISA kit and Conditions of Storage

Item	Unit	State	Amt.	Storage Conditions
Tween TM 20	1 vial	liquid	250 µL/vial	4°C
H ₂ O ₂	1 vial	liquid	250 µL/vial	4°C
(s)-ibuprofen-HRP	1 vial	liquid	250 µL/vial	4°C
2-carboxyibuprofen HRP	1 vial	liquid	250 µL/vial	4°C
Buffer A	4 vials	Solid	0.8894 g/vial	4°C
Buffer B	6 vials	Solid	1.234 g/vial	4°C
Buffer C	6 vials	Solid	1.1170 g/vial	4°C
Buffer D	6 vials	Solid	0.8082 g/vial	4°C
Plate ((s)-ibuprofen-Ab)	2	Solid	-	4°C
Plate (2-carboxyibuprofen - Ab)	2	Solid	-	4°C
Buffer E	6 vials	Solid	0.9567 g/vial	-20°C
Standards ((s)-ibuprofen)	14 vials	Liquid	200 µL	-20°C
Standards (2- carboxyibuprofen)	14 vials	Liquid	200 µL	-20°C
1N NaOH	1 bottle	Liquid	15 mL	20°C
1N HCl	1 bottle	Liquid	15 mL	20°C

* Composition of all buffers is described in Table 10

Solutions

Buffer A: Dissolve the content of 1 vial A/ 25mL water.

Buffer B: Dissolve the content of 1 vial B/ 100mL water.

Buffer C: Dissolve the content of one vial C/50 mL water. Add 25 mL of TweenTM 20.

Buffer D: Dissolve the content of one vial D/25 mL water. Add 25 mL of TweenTM 20.

0.05% TweenTM 20: Add 25 mL of TweenTM 20 to a 100 mL erlenmeyer flask containing 50 mL of water.

2.5N HCl: 41.75 mL of 12 N HCl/200 mL water.
Store in a 250 mL glass bottle.

(s)-ibuprofen-HRP conjugate: Add 9 mL of Buffer C to a 15 mL glass test tube. Add 90 µL of (s)-ibuprofen-HRP stock solution.

2-carboxyibuprofen-HRP conjugate: Add 9 mL of Buffer C to a 15 mL glass test tube. Add 90 µL of 2-carboxyibuprofen-HRP stock solution.

Buffer E - H₂O₂: Dissolve the contents of 1 vial E-substrate/50 mL water. Add 25 µL of a 30% H₂O₂ solution (prepared fresh).

Dilutions of Urine Samples for the Determinations of [(s)-ibuprofen-HRP] and [2-carboxyibuprofen-HRP] by ELISA

The dilutions of urine samples required for determinations of (s)-ibuprofen and 2-carboxyibuprofen are a function of the sensitivity of the competitive antigen ELISA and of (s)-ibuprofen and 2-
5 carboxyibuprofen concentrations in urine samples. It is suggested to dilute the urine samples by a factor so (s)-ibuprofen and 2-carboxyibuprofen are about 3×10^{-6} M in the well of the microtiter plate (see table 4).

Table 4
Microtube #

Dilution Factor	20×	40×	50×	80×	100×	150×	200×	400×
Solution	1	2	3	4	5	6	7	8
Urine Sample (μL) ^a 10× diluted	500	250	200	125	100	66.7	50	25
Buffer B (μL)	500	750	800	875	900	933.3	950	975

a: Vortex the microtubes containing the urine sample before pipetting.

Store the diluted urine samples at -20°C in a box for microtubes.

Determination of [(s)-ibuprofen] and [2-carboxyibuprofen] in Diluted Urine Samples by ELISA

Precautions

The HRP substrate (p-nitrophenolphosphate) is carcinogenic. Wear surgical gloves when handling Buffer E (substrate buffer). Each sample is determined in duplicate. An excellent pipetting technique is required. When this technique is mastered the absorbency values of duplicates should be within less than 5%. Buffers C, D, E are freshly prepared. Buffer E-H₂O₂ is prepared just prior to pipetting in the microtiter plate wells.

Preparation of Samples

Table 5 is prepared with a computer and printed. This table shows the contents of each well of a 96 well microtiter plate. The name of the urine sample (or number) is entered at the corresponding well positions in Table 5. The dilution factor (D.F.) of each urine sample is selected and entered at the corresponding position in Table 5. The dilution of each urine sample with buffer B is entered at the corresponding position in Table 5: for example, for a D.F. of 100 (100 μ L of 10 $\times$ diluted urine sample + 900 μ L buffer B), 100/900 is entered. See "Dilutions of Urine Samples..." procedure described above for the preparation of the different dilutions. The different dilutions of the urine samples are prepared in 1.5 mL microtubes using a styrofoam support for 100 microtubes. Standard solutions of concentrations indicated in Table 6 are preferably provided with the kit of the present invention. Table 7 is prepared with a computer and printed. Using a styrofoam support (100 microtubes), the following 48 microtubes are prepared in the order as indicated in Table 7.

Table 5
Positions of Blanks, Control and Urine Samples in a Microtiter Plate

Sample	Well #	D.F.	Dil.	Sample	Well #	D.F.	Dil.
Blank	1-2	-		Control	49-50		-
Control	3-4	-		8	51-52		
S1	5-6	-		9	53-54		
S2	7-8	-		10	55-56		
S3	9-10	-		11	57-58		
S4	11-12	-		12	59-60		
S5	13-14	-		13	61-62		
S6	15-16	-		14	63-64		
S7	17-18	-		15	65-66		
S8	19-20	-		16	67-68		
S9	21-22	-		17	69-70		
S10	23-24	-		Control	71-72		-
S11	25-26	-		18	73-74		
S12	27-28	-		19	75-76		
S13	29-30	-		20	77-78		
S14	31-32	-		21	79-80		
S15	33-34	-		22	81-82		
1	35-36			23	83-84		
2	37-38			24	85-86		
3	39-40			25	87-88		
4	41-42			26	89-90		
5	43-44			27	91-92		
6	45-46			28	93-94		
7	47-48			Blank	95-96		-

Table 6

Standard Solutions of (s)-ibuprofen and 2-carboxyibuprofen (Diluted with Buffer B)

Standard	(s)-ibuprofen	Standard	2-carboxyibuprofen
1	$1.12 \times 10^{-4} \text{ M}$	1	$1.12 \times 10^{-4} \text{ M}$
2	$6.00 \times 10^{-5} \text{ M}$	2	$6.00 \times 10^{-5} \text{ M}$
3	$3.56 \times 10^{-5} \text{ M}$	3	$3.56 \times 10^{-5} \text{ M}$
4	$2.00 \times 10^{-5} \text{ M}$	4	$2.00 \times 10^{-5} \text{ M}$
5	$6.00 \times 10^{-6} \text{ M}$	5	$6.00 \times 10^{-6} \text{ M}$
6	$3.56 \times 10^{-6} \text{ M}$	6	$3.56 \times 10^{-6} \text{ M}$
7	$2.00 \times 10^{-6} \text{ M}$	7	$2.00 \times 10^{-6} \text{ M}$
8	$1.12 \times 10^{-6} \text{ M}$	8	$1.12 \times 10^{-6} \text{ M}$
9	$6.00 \times 10^{-7} \text{ M}$	9	$6.00 \times 10^{-7} \text{ M}$
10	$3.56 \times 10^{-7} \text{ M}$	10	$3.56 \times 10^{-7} \text{ M}$
11	$2.00 \times 10^{-7} \text{ M}$	11	$2.00 \times 10^{-7} \text{ M}$
12	$1.12 \times 10^{-7} \text{ M}$	12	$1.12 \times 10^{-7} \text{ M}$
13	$6.00 \times 10^{-8} \text{ M}$	13	$6.00 \times 10^{-8} \text{ M}$
14	$3.56 \times 10^{-8} \text{ M}$	14	$3.56 \times 10^{-8} \text{ M}$
15	$2.00 \times 10^{-8} \text{ M}$	15	$2.00 \times 10^{-8} \text{ M}$

Table 7
Content of Microtubes for CYP 2C9 phenotyping ELISA

Tube #	Sample	Content	Tube #	Sample	Content
1	Blank	Buffer B	25	7	Dil. Urine
2	Control	Buffer B	26	8	Dil. Urine
3	S1	(s)-ibuprofen/2-carboxyibuprofen	27	9	Dil. Urine
4	S2	(s)-ibuprofen/2-carboxyibuprofen	28	10	Dil. Urine
5	S3	(s)-ibuprofen/2-carboxyibuprofen	29	11	Dil. Urine
6	S4	(s)-ibuprofen/2-carboxyibuprofen	30	12	Dil. Urine
7	S5	(s)-ibuprofen/2-carboxyibuprofen	31	13	Dil. Urine
8	S6	(s)-ibuprofen/2-carboxyibuprofen	32	14	Dil. Urine
9	S7	(s)-ibuprofen/2-carboxyibuprofen	33	15	Dil. Urine
10	S8	(s)-ibuprofen/2-carboxyibuprofen	34	16	Dil. Urine
11	S9	((s)-ibuprofen/2-carboxyibuprofen	35	17	Dil. Urine
12	S10	(s)-ibuprofen/2-carboxyibuprofen	36	Control	Buffer B
13	S11	(s)-ibuprofen/2-carboxyibuprofen	37	18	Dil. Urine
14	S12	(s)-ibuprofen/2-carboxyibuprofen	38	19	Dil. Urine
15	S13	(s)-ibuprofen/2-carboxyibuprofen	39	20	Dil. Urine
16	S14	(s)-ibuprofen/2-carboxyibuprofen	40	21	Dil. Urine
17	S15	(s)-ibuprofen/2-carboxyibuprofen	41	22	Dil. Urine
18	1	Dil. Urine	42	23	Dil. Urine
19	2	Dil. Urine	43	24	Dil. Urine
20	3	Dil. Urine	44	25	Dil. Urine
21	4	Dil. Urine	45	26	Dil. Urine
22	5	Dil. Urine	46	27	Dil. Urine
23	6	Dil. Urine	47	28	Dil. Urine
24	Control	Buffer B	48	Blank	Buffer B

Conditions of the ELISA

Starting from the last row, 50 μ L/well of (s)-ibuprofen-HRP ((s)-ibuprofen/2-carboxyibuprofen) conjugate are added. Next are added 50 μ L/well of diluted urine samples in duplicate, standards, and

blanks with a micropipet (0-200 μ L), starting from well # 96 (see Table 8). The plate is covered and mixed gently by vortexing for several seconds. The plate is left at room temperature for 3 h. Then, the wells are
5 washed 3 times with 100 μ L/well Buffer C, using a microtiter plate washer. The wells are then washed 3 times with 100 μ L/well 0.05% TweenTM-20 solution. Next, 150 μ L/well of Buffer E- H₂O₂ (prepared just prior to pipetting in the microtiter plate wells) are added.
10 The plate is shaken for 20-30 min at room temperature using an orbital shaker. After shaking, 50 μ L/well of a 2.5N HCl solution are added. The plate is shaken again 3 min with the orbital shaker at room temperature. The absorbance of the wells are read with
15 a microtiter plate reader at 490 nm. The sheet of data is printed and properly labelled.

Calculation of the [(s)-ibuprofen] and [2-carboxyibuprofen] in Urine Samples from the Data

Table 8 is drawn with a computer. Using the data sheet of the microtiter plate reader, the average
20 absorbance values of blanks, controls (no free hapten present), standards and samples are entered in Table 8.

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The calibration curve is drawn on a semi-logarithmic plot (absorbance at 490 nm as a function of the standard concentrations) using sigma-plot (or other plot software). The [(s)-ibuprofen] (or [2-carboxyibuprofen]) is found in the microtiter well of the unknowns from the calibration curve and entered in the data in Table 9. The [(s)-ibuprofen] (or [2-carboxyibuprofen]) of the unknown is multiplied by the dilution factor and the result is entered in the corresponding cell of Table 9.

Table 8
Average Absorbance Values of Samples in the Microtiter Plate

Sample	Well #	A ₄₉₀	Sample	Well #	A ₄₉₀
Blank	1-2		Control	49-50	
Control	3-4		8	51-52	
S1	5-6		9	53-54	
S2	7-8		10	55-56	
S3	9-10		11	57-58	
S4	11-12		12	59-60	
S5	13-14		13	61-62	
S6	15-16		14	63-64	
S7	17-18		15	65-66	
S8	19-20		16	67-68	
S9	21-22		17	69-70	
S10	23-24		Control	71-72	
S11	25-26		18	73-74	
S12	27-28		19	75-76	
S13	29-30		20	77-78	
S14	31-32		21	79-80	
S15	33-34		22	81-82	
1	35-36		23	83-84	
2	37-38		24	85-86	
3	39-40		25	87-88	
4	41-42		26	89-90	
5	43-44		27	91-92	
6	45-46		28	93-94	
7	47-48		Blank	95-96	

Table 9
(s)-ibuprofen and 2-carboxyibuprofen Concentrations
in Urine Samples

Sample	D.F.	[(s)-ibuprofen]	[(s)-ibuprofen] × D.F.
1			
2			
3			
4			
5			
6			
7			
8			
9			
10			
11			
12			
13			
14			
15			
16			
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19			
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27			
28			
29			

Table 10
Composition of the Different Buffers

Buffer	pH	Composition	Conc. (mM)	[P] (mM)
A	7.50	0.15629 g/100 mL NaH_2PO_4 1.622 g/100 mL $\text{Na}_2\text{HPO}_4 \cdot 7\text{H}_2\text{O}$ 1.778 g/100 mL (total weight)	11.325 60.099	71.424
B	7.50	0.1210191 g/100 mL NaH_2PO_4 1.11309 g/100 mL $\text{Na}_2\text{HPO}_4 \cdot 7\text{H}_2\text{O}$ 1.2341 g/100 mL (total weight)	8.769 41.23	49.999
C	7.50	1 g/ 100mL BSA 0.1210191 g/100 mL NaH_2PO_4 1.11309 g/100 mL $\text{Na}_2\text{HPO}_4 \cdot 7\text{H}_2\text{O}$ 2.2341 g/100 mL (total weight)	8.769 41.23	49.999
D	7.50	2 g/ 100mL BSA 0.1210191 g/100 mL NaH_2PO_4 1.11309 g/100 mL $\text{Na}_2\text{HPO}_4 \cdot 7\text{H}_2\text{O}$ 3.2341 g/100 mL (total weight)	8.769 41.23	49.999
E	5.00	0.52508 g/ 100mL of citric acid 1.34848 g/100 mL $\text{Na}_2\text{HPO}_4 \cdot 7\text{H}_2\text{O}$ 40 mg/100 mL of o-phenylenedi- amine hydrochloride 1.913567 g/100 mL (total weight)	25 50	-

DISCUSSION

In the form of a kit, the present invention provides a convenient and effective tool for use in both a clinical and laboratory environment. The kit of the present invention is particularly suited for use by
5 a physician or other qualified personnel in a clinic,

whereby a fast and accurate result can be easily obtained. According to an embodiment of the present invention, a ready-to-use kit is provided for fast and accurate determination of an individual's CYP 2C9
5 phenotype. Preferably, a kit of the present invention includes a microtest plate having a plurality of wells for receiving biological samples to be tested for metabolite concentrations indicative of a CYP 2C9-specific phenotypic determinant. The microtest plate
10 may be pre-bound with antibodies specific to the metabolites of interest. The kit may further include suitable substrates and buffers, such as those exemplified in Table 3.

As a result of the convenience and ease of use
15 of ELISA and/or kit of the present invention, a physician is provided with a tool for use in the individualization of treatment. A quick and accurate determination of an individual's CYP 2C9 phenotype will allow a physician to consider this information before
20 prescribing a treatment regime. In this manner, a method of individualizing treatment is also provided. In essence, a CYP 2C9 phenotype characterization, according to the present invention, can serve as a drug response profile specific to drugs known to be

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metabolized by CYP 2C9 for the individual phenotyped. Furthermore, the ELISA and/or kit of the present invention may be used to screen individuals for their susceptibility to carcinogens or for their phenotypic compatibility with a particular drug known to metabolized completely or in part by CYP 2C9.

The present invention provides a convenient and effective tool for use in both a clinical and laboratory environment. The present invention is particularly suited for use by a physician in a clinic, whereby phenotypic determinants of CYP 2C9 can be quickly and easily obtained. According to an embodiment of the present invention, a ready-to-use kit is provided for fast and accurate determination of at least CYP 2C9 determinants. The assay system and kit preferably employ antibodies specific to a plurality of substrates and/or forms thereof on a suitable substrate allowing for detection of the preferred substrates in a biological sample of an individual after consumption of a corresponding substrate (or probe substrate). In accordance with a preferred embodiment of the present invention, the kit of the present invention will provide means to determine metabolic determinants for at least CYP 2C9. The assay system and method of the

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present invention may be provided in a plurality of forms including but not limited to an ELISA assay, a high-throughput ELISA assay or a dipstick based ELISA assay.

5 The ELISA and/or kit of an embodiment of the present invention includes antibodies specific to preferred metabolites, substrates and/or forms thereof known to be acted on by the CYP 2C9 metabolic pathway immobilized on a suitable substrate to detect the
10 presence of the preferred metabolites, substrates and/or forms thereof in a biological sample of an individual after consumption of a corresponding probe substrate.

 While the invention has been described in
15 connection with specific embodiments thereof, it will be understood that it is capable of further modifications and this application is intended to cover any variations, uses, or adaptations of the invention following, in general, the principles of the invention
20 and including such departures from the present disclosure as come within known or customary practice within the art to which the invention pertains and as may be applied to the essential features hereinbefore

set forth, and as follows in the scope of the appended claims.

All references cited within this application are hereby incorporated by reference.

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WHAT IS CLAIMED IS:

1. A method of characterizing a CYP 2C9-specific phenotype, said method comprising:

- a) administering to an individual a probe substrate known to be metabolized by a CYP 2C9 metabolic pathway;
- b) detecting concentrations of said probe substrate and/or forms thereof in a biological sample obtained from said individual at a predetermined time after said administering of said probe substrate; and
- c) characterizing a phenotypic determinant based on said concentrations of said probe substrate and/or forms thereof which is indicative of said CYP 2C9 phenotype.

2. The method of claim 1 wherein said probe substrate is (s)-ibuprofen.

3. The method of claim 2 wherein said probe substrate and/or forms of said probe substrate include (s)-ibuprofen and 2-carboxyibuprofen.

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4. The method of claim 3 wherein said phenotypic determinant is characterized according to a molar ratio of concentrations of said probe substrate and/or forms of said probe substrate, as calculated by:

$$\frac{[(s)\text{-ibuprofen}]}{[2\text{-carboxyibuprofen}]}.$$

5. The method of claim 1, wherein said step of detecting concentrations of said probe substrate and/or forms thereof includes a ligand-binding assay, whereby said assay includes treating said biological sample with binding molecules specific to each of said probe substrates and/or forms thereof.

6. The method of claim 5, wherein said step of detecting concentrations of said probe substrate and/or forms thereof further includes measuring the absorbency of a binding molecule-ligand conjugate for each of said probe substrate and/or forms thereof.

7. The method of claim 5, wherein said binding molecules specific to each of said probe substrates and/or forms thereof are antibodies.

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8. The method of claim 1, wherein said biological sample is a urine sample.

9. The method of claim 1, wherein said phenotypic determinant is indicative of said individual's susceptibility to a carcinogen induced disease.

10. The method of claim 9, wherein said carcinogen-induced disease is cancer.

11. The method of claim 1, wherein said phenotypic determinant is indicative of said individual's responsiveness to a drug known to be metabolized by CYP 2C9.

12. The method of claim 1 for use in selecting a drug treatment regime for said individual.

13. The method of claim 1 for use in screening individuals for a CYP 2C9 phenotype requirement prior to participation in a clinical trial.

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14. The method of claim 1 for use in individualization of treatment wherein said treatment is influenced by CYP 2C9 metabolism.

15. A competitive enzyme linked immunosorbent assay (ELISA) method for determining a CYP 2C9 phenotype, which comprises using at least two antibodies specific to (s)-ibuprofen and 2-carboxyibuprofen, respectively, to determine the amount of each of (s)-ibuprofen and 2-carboxyibuprofen in a biological sample obtained from an individual treated with (s)-ibuprofen; wherein a molar ratio based on amounts of the (s)-ibuprofen to 2-carboxyibuprofen is indicative of a CYP 2C9 phenotype of said individual.

16. The ELISA method of claim 15, wherein said biological sample is a urine sample.

17. The ELISA method of claim 15, wherein said determined CYP 2C9 phenotype of said individual provides an indication of said individual's susceptibility to a carcinogen-induced disease.

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18. The method of claim 15, wherein said disease is cancer.

19. The ELISA method of claim 15, wherein said CYP 2C9 phenotype provides a drug response profile for said individual.

20. The method of claim 18 for use in selecting a drug treatment regime for said individual.

21. A competitive enzyme linked immunosorbent assay (ELISA) kit for determining a CYP 2C9 phenotype of an individual, which comprises at least two antibodies each specific to a probe substrate and/or at least one other form thereof for detecting the molar ratio of said probe substrate and/or at least one other form thereof in a biological sample of an individual after consuming a dose of said probe substrate wherein said molar ratio is indicative of said CYP 2C9 phenotype.

22. The competitive enzyme linked immunosorbent assay (ELISA) kit of claim 21 for determining a CYP 2C9 phenotype of an individual, said kit comprising at

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least one plate having a plurality of microwells for receiving biological samples obtained from said individual; said microwells having an antibody coating selected from said at least two antibodies.

23. The competitive enzyme linked immunosorbent assay (ELISA) kit of claim 21 wherein said at least two antibodies are specific to (s)-ibuprofen and 2-carboxyibuprofen, respectively.

24. The competitive enzyme linked immunosorbent assay (ELISA) kit of claim 21, further comprising:

- a) a known amount of (s)-ibuprofen-horseradish peroxidase conjugate wherein a standard calibration curve is obtained; and
- b) a known amount of 2-carboxyibuprofen-horseradish peroxidase conjugate wherein a standard calibration curve is obtained.

25. The kit of claim 21, for use in determining the susceptibility of said individual to a carcinogen-induced disease.

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26. The kit of claim 21, for use in determining a drug response profile specific to said individual.

27. The kit of claim 21, for use in selecting a drug treatment regime for said individual.

28. The method of claim 5, wherein said binding molecules are monoclonal antibodies.

29. The method of claim 5, wherein said binding molecules are polyclonal antibodies.

30. The competitive antigen enzyme linked immunosorbent assay (ELISA) method of claim 15 wherein said at least two antibodies are monoclonal antibodies.

31. The competitive antigen enzyme linked immunosorbent assay (ELISA) method of claim 15 wherein said at least two antibodies are polyclonal antibodies.

32. The competitive ELISA kit of claim 21 wherein said at least two antibodies are monoclonal antibodies.

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33. The competitive ELISA kit of claim 21 wherein said at least two antibodies are polyclonal antibodies.

34. A (s)-ibuprofen derivative as illustrated in Fig. 3.

35. A 2-carboxyibuprofen derivative as illustrated in Fig. 4.

36. The (s)-ibuprofen derivative of claim 34 for use in raising antibodies having an affinity for (s)-ibuprofen.

37. The 2-carboxyibuprofen derivative of claim 35 for use in raising antibodies having an affinity for 2-carboxyibuprofen.

38. The (s)-ibuprofen derivative of claim 34 for use in detecting (s)-ibuprofen in a biological sample.

39. The 2-carboxyibuprofen derivative of claim 35 for use in detecting 2-carboxyibuprofen in a biological sample.

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40. The antibodies of claim 36 for use in an ELISA for determining a CYP 2C9 phenotype.

41. The antibodies of claim 37 for use in an ELISA for determining a CYP 2C9 phenotype.

42. The method of claim 1 wherein said probe substrate is losartan.

43. The method of claim 2 wherein said probe substrate and/or forms of said probe substrate include losartan and E-3174.

44. The method of claim 3 wherein said phenotypic determinant is characterized according to a molar ratio of concentrations of said probe substrate and/or forms of said probe substrate, as calculated by:

$$\frac{[\text{losartan}]}{[\text{E-3174}]}.$$

45. A competitive enzyme linked immunosorbent assay (ELISA) method for determining a CYP 2C9 phenotype, which comprises using at least two antibodies specific to losartan and E-3174, respectively, to determine the

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amount of each of losartan and E-3174 in a biological sample obtained from an individual treated with losartan; wherein a molar ratio based on amounts of the losartan to losartan is indicative of a CYP 2C9 phenotype of said individual.

46. The ELISA method of claim 45, wherein said biological sample is a urine sample.

47. The ELISA method of claim 45, wherein said determined CYP 2C9 phenotype of said individual provides an indication of said individual's susceptibility to a carcinogen-induced disease.

48. The method of claim 45, wherein said disease is cancer.

49. The ELISA method of claim 45, wherein said CYP 2C9 phenotype provides a drug response profile for said individual.

50. The method of claim 48 for use in selecting a drug treatment regime for said individual.

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51. The competitive enzyme linked immunosorbent assay (ELISA) kit of claim 21 wherein said at least two antibodies are specific to losartan and E-3174, respectively.

52. The competitive enzyme linked immunosorbent assay (ELISA) kit of claim 21, further comprising:

- a) a known amount of losartan-horseradish peroxidase conjugate wherein a standard calibration curve is obtained; and
- b) a known amount of E-3174-horseradish peroxidase conjugate wherein a standard calibration curve is obtained.

53. The competitive antigen enzyme linked immunosorbent assay (ELISA) method of claim 45 wherein said at least two antibodies are monoclonal antibodies.

54. The competitive antigen enzyme linked immunosorbent assay (ELISA) method of claim 45 wherein said at least two antibodies are polyclonal antibodies.

55. A losartan derivative as illustrated in Fig. 5.

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56. A E-3174 derivative as illustrated in Fig. 6.

57. The losartan derivative of claim 55 for use in raising antibodies having an affinity for losartan.

58. The E-3174 derivative of claim 56 for use in raising antibodies having an affinity for E-3174.

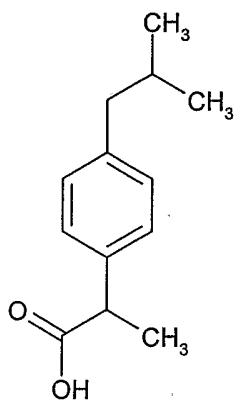
59. The losartan derivative of claim 55 for use in detecting losartan in a biological sample.

60. The E-3174 derivative of claim 56 for use in detecting E-3174 in a biological sample.

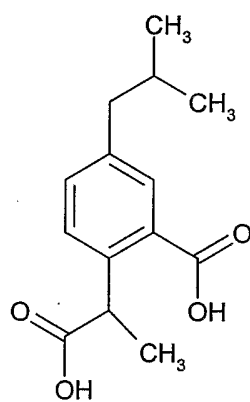
61. The antibodies of claim 57 for use in an ELISA for determining a CYP 2C9 phenotype.

62. The antibodies of claim 58 for use in an ELISA for determining a CYP 2C9 phenotype.

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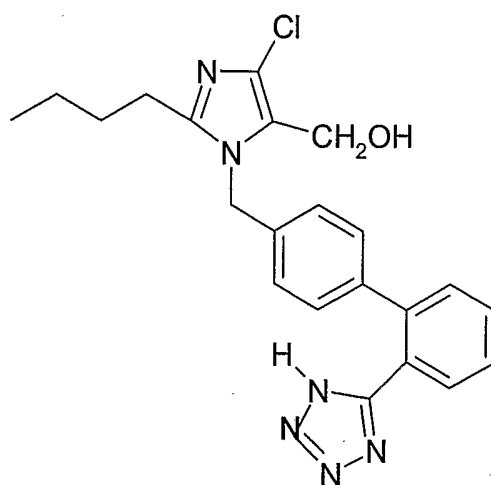
(s) -Ibuprofen



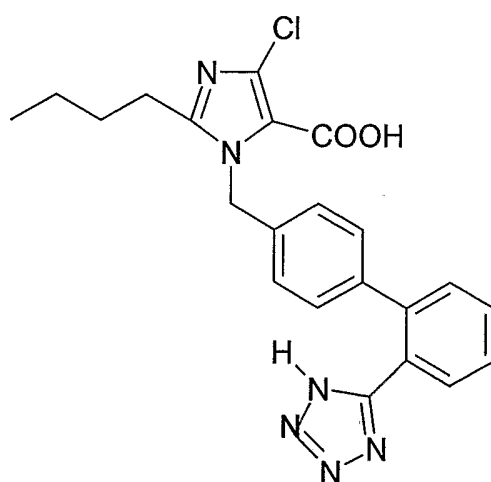
2-carboxyibuprofen

Fig. 1

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Losartan

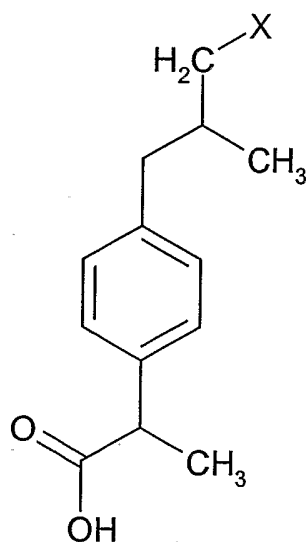


E-3174

Fig. 2

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(s)-ibuprofen derivatives for CYP2C9
phenotyping by ELISA



Where X=

$(\text{CH}_2)_n\text{COOH}$ $n=1-5$

OR

$(\text{CH}_2)_n\text{NH}_2$ $n=1-5$

OR

$(\text{CH}_2)_n\text{OH}$ $n=2-5$

OR

$(\text{CH}_2)_n\text{NHCOOCH}_2\text{CH}_2\text{COOH}$

$n=1-5$

OR

$(\text{CH}_2)_n\text{OCOCH}_2\text{CH}_2\text{COOH}$ $n=1-5$

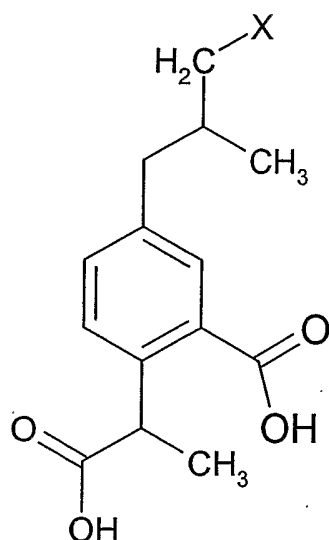
OR

$(\text{CH}_2)_n\text{N}=\text{C}=\text{S}$ $N=1-5$

Fig 3

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2-carboxyibuprofen derivatives for CYP2C9
phenotyping by ELISA



Where X=

$(\text{CH}_2)_n\text{COOH}$ $n=1-5$

OR

$(\text{CH}_2)_n\text{NH}_2$ $n=1-5$

OR

$(\text{CH}_2)_n\text{OH}$ $n=2-5$

OR

$(\text{CH}_2)_n\text{NHCOOCH}_2\text{CH}_2\text{COOH}$

$n=1-5$

OR

$(\text{CH}_2)_n\text{OCOCH}_2\text{CH}_2\text{COOH}$ $n=1-5$

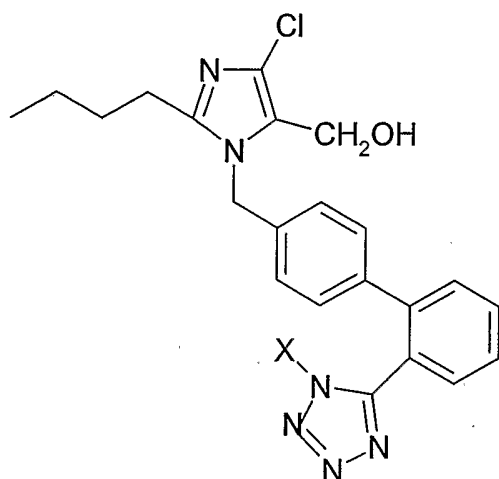
OR

$(\text{CH}_2)_n\text{N}=\text{C}=\text{S}$ $N=1-5$

Fig. 4

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Losartan derivatives for CYP2C9 phenotyping
by ELISA



Where X=

$(\text{CH}_2)_n\text{COOH}$ $n=1-5$

OR

$(\text{CH}_2)_n\text{NH}_2$ $n=1-5$

OR

$(\text{CH}_2)_n\text{OH}$ $n=2-5$

OR

$(\text{CH}_2)_n\text{NHCOOCH}_2\text{CH}_2\text{COOH}$

$n=1-5$

OR

$(\text{CH}_2)_n\text{OCOCH}_2\text{CH}_2\text{COOH}$ $n=1-5$

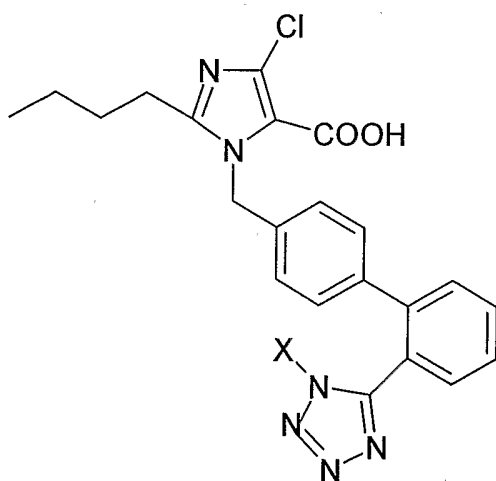
OR

$(\text{CH}_2)_n\text{N}=\text{C}=\text{S}$ $n=1-5$

Fig. 5

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E-3174 derivatives for CYP2C9 phenotyping
by ELISA



Where X=

$(\text{CH}_2)_n\text{COOH}$ $n=1-5$

OR

$(\text{CH}_2)_n\text{NH}_2$ $n=1-5$

OR

$(\text{CH}_2)_n\text{OH}$ $n=2-5$

OR

$(\text{CH}_2)_n\text{NHCOOCH}_2\text{CH}_2\text{COOH}$
 $n=1-5$

OR

$(\text{CH}_2)_n\text{OCOCH}_2\text{CH}_2\text{COOH}$ $n=1-5$

OR

$(\text{CH}_2)_n\text{N}=\text{C}=\text{S}$ $n=1-5$

Fig. 6

	1	2	3	4	5	6	7	8	9	10	11	12
A	Blk	STD8	STD16	S1	S9	S5	S1	S9	S5	Blk	STD8	STD16
B	STD1	STD9	STD17	S2	S10	S6	S2	S10	S6	STD1	STD9	STD17
C	STD2	STD10	STD18	S3	S11	S7	S3	S11	S7	STD2	STD10	STD18
D	STD3	STD11	STD19	S4	S12	S8	S4	S12	S8	STD3	STD11	STD19
E	STD4	STD12	STD20	S5	S1	S9	S5	S1	S9	STD4	STD12	STD20
F	STD5	STD13	STD21	S6	S2	S10	S6	S2	S10	STD5	STD13	STD21
G	STD6	STD14	STD22	S7	S3	S11	S7	S3	S11	STD6	STD14	STD22
H	STD7	STD15	STD23	S8	S4	S12	S8	S4	S12	STD7	STD15	STD23

Fig. 7