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Monolithic silica rod liquid chromatography with ultraviolet or fluorescence detection for metabolite analysis of cytochrome P450 marker reactions

E.S.M. Lutz*, M.E. Markling, C.M. Masimirembwa

DMPK & Bioanalytical Chemistry, AstraZeneca R&D Mölndal, S-431 83 Mölndal, Sweden

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Abstract

In vitro cytochrome P450 assays are used in metabolism studies in support of early phases of drug discovery to investigate, e.g., metabolic stability, enzyme inhibition and induction by new chemical entities. LC–UV and LC–fluorescence are traditional analytical tools in support of such studies. However, these tools typically comprise different methods of relatively low throughput for the various metabolites of probe reactions. In recent years, LC–MS methods have been developed to increase throughput. Increased throughput can also be achieved by means of modern chromatographic tools in combination with UV and fluorescence detection. This approach is especially suitable when cytochrome P450 isoforms are investigated by means of single probe incubations. Here, an LC–UV/fluorescence system based on a monolithic porous silica column is described for the analysis of metabolites of nine cytochrome P450 marker reactions [phenacetin to paracetamol (CYP1A2), coumarin to 7-hydroxycoumarin (CYP2A6), paclitaxel to 6α-hydroxypaclitaxel (CYP2C8), diclofenac to 4-hydroxydiclofenac (CYP2C9), mephenytoin to 4-hydroxymephenytoin (CYP2C19), bufuralol to 1-hydroxybufuralol (CYP2D6), chlorzoxazone to 6-hydroxychlorzoxazone (CYP2E1), midazolam to 1-hydroxymidazolam (CYP3A4), and testosteron to 6β-hydroxytestosteron (CYP3A4)]. While offering sensitivities and linear ranges comparable to previously reported methods, the set-up described here provides ease of use and increased throughput with maximum cycle times of 4.5 min.

Keywords: Monolithic silica rod; Cytochrome P450

1. Introduction

Drug metabolism plays an important role in drug disposition, and thus influences pharmacology and toxicology of therapeutic compounds. A major enzyme system involved in drug metabolism is cytochrome P450. This is a superfamily of mixed function oxidases with a common catalytic centre comprising a conserved haem–thiolate functionality, but different three-dimensional structures at the active site due to amino acid variations entailing substrate specificity of the individual cytochrome P450 isozymes [1]. In vitro cytochrome P450 metabolism studies of new chemical entities (NCEs) are aimed at understanding metabolic parameters during early stages of drug discovery and include, e.g., metabolic stability, identification of specific enzymes involved

*Corresponding author. Fax: +46-31-776-3748.
E-mail address: mareike.lutz@astrazeneca.com (E.S.M. Lutz).

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in the NCE's metabolism, and assessment of inhibitory and inductory effects [2].

Assessment of inhibitory effects is evaluated in order to predict drug–drug interactions involving a NCE. It can be monitored by the NCE's effect on the metabolism of compounds which are metabolized specifically by one cytochrome P450 isoform. Such compounds are referred to as probe drugs, their enzymatic conversion by the cytochrome P450 a marker reaction. Marker reactions in the presence of an NCE are widely studied in vitro using human liver preparations, such as hepatocytes, liver slices, and liver microsomes [3]. In our laboratory, mainly human liver microsomes are used for routine investigations employing the marker reactions listed in Fig. 1.

Traditional analytical support in metabolism studies with human liver microsome assays consists of liquid chromatography (LC)–UV and LC–fluorescence. Numerous LC–UV and LC–fluorescence methods have been described in the literature, and have been compiled, among others, by Masimirembwa et al. [4], Ayrton et al. [5], and Philips and Shephard [6]. However, these traditional tools typically provide a relatively low throughput, and when analysing various marker reactions on a single LC system, practical issues involving system reconfiguration need to be considered [5,7,8].

Following the constant call for increased analytical throughput, various approaches are feasible to strengthen in vitro cytochrome P450 research. The advent of LC–mass spectrometry (MS) has enabled the use of so-called cocktail assays, i.e., monitoring of several cytochrome P450 enzymes by simultaneous incubation of marker substrates [5,7,8]. Alternatively, assays based on single substrate incubations can be pooled prior to LC–MS analysis [9]. In principle, other approaches developed for quantitative bioanalysis of pharmaceutical compounds can also be applied to increase throughput, such as turbulent flow LC [10–12], parallel sample processing by means of multiplexed electrospray interface (MUX) [13,14], etc. All these approaches rely, in principle, on selective detection by mass spectrometry.

When metabolic investigations are directed to investigating single cytochrome P450 isoforms by means of single marker substrate incubations, LC–MS is not required for analysis. For such applications, LC–UV and LC–fluorescence are valuable tools that reduce the strain on advanced analytical resources.

The approach described in this paper consists of a conventional LC–UV/fluorescence set-up employing a monolithic porous silica column instead of a conventional particulate column. The advantageous feature of the monolithic column is the reduced pressure drop in comparison to a particulate column while maintaining separation efficiencies. This is due to the biporous structure of the monolithic column which consists of macropores (2 μm) and mesopores (13 nm). The macropores allow high flow-rates due to higher porosity and thus offer reduced flow resistance, whereas the mesopores provide the surface area for achieving sufficient capacity [15–20]. The LC–UV/fluorescence set-up is described for analysing single substrate cytochrome P450 marker reactions currently used in our laboratory.

2. Experimental

2.1. Chemicals

Phenacetin, coumarin, diclofenac, paclitaxel, chlorzoxazone, 7-hydroxycoumarin and 4-acetamidophenol (paracetamol) were obtained from Sigma (St. Louis, MO, USA), *S*-mephenytoin, bufuralol, 4-hydroxymephenytoin, 1-hydroxymidazolam, 6-hydroxychlorzoxazone, 1-hydroxybufuralol maleate salt from Ultrafine (Manchester, UK), midazolam, 6α-hydroxypaclitaxel, 4-hydroxydiclofenac from Gentest (Woburn, MA, USA), testosteron and 6β-hydroxytestosteron from Steraloids (Newport, RI, USA).

Acetonitrile was purchased from Rathburn (Walkerburn, UK). Potassium dihydrogenphosphate-3-hydrate, dipotassium hydrogenphosphate and phosphoric acid were obtained from Merck (Darmstadt, Germany). Deionised water was produced in an ELGA purification system (High Wycombe, UK).

2.2. Human liver microsomes

Human liver microsomes (HLMs) were prepared from pooled liver pieces from patients undergoing

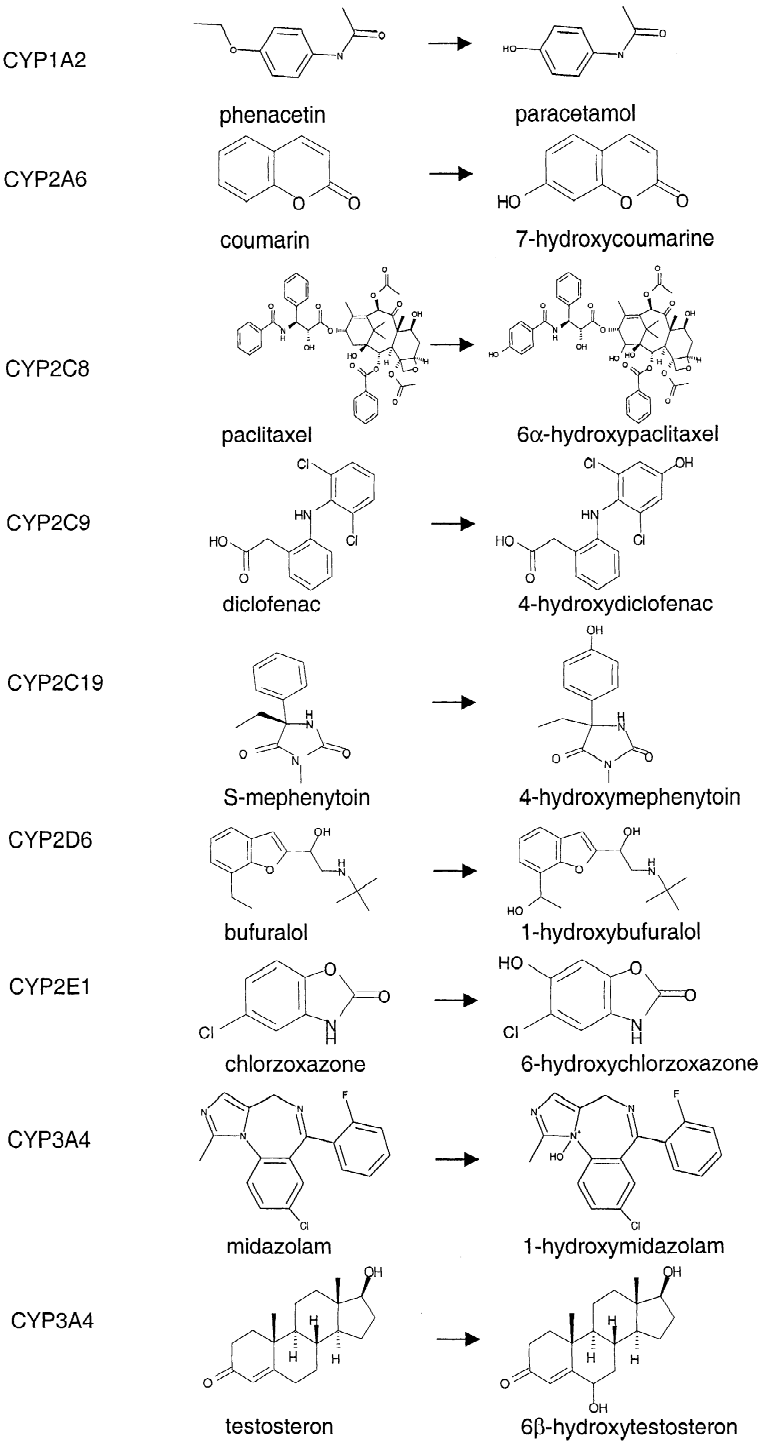


Fig. 1. Chemical structures of the substrates and metabolites of the cytochrome P450 marker reactions.

liver resections according to the method of Pearce et al. [21].

2.3. Assay conditions

All reactions were performed in total volumes of 200 μl. The incubation mixtures consisted of the marker substrate at K_m concentrations (Table 1) [22], i.e., the concentration of substrate which result in half the maximal reaction velocity, in 5 μl acetonitrile, 0.1 mol/l potassium phosphate buffer at pH 7.4, 0.2 mg/ml HLMs. After 10 min preincubation, the reactions were started by adding 1 mmol/l NADPH and incubated at 37 °C. Reactions were stopped after incubation for 20 min at 37 °C by addition of 100 μl cold methanol, the CYP2C9 marker reaction (diclofenac to 4-hydroxydiclofenac) was stopped by addition of 100 μl cold methanol and 10 μl 3 mol/l phosphoric acid. Samples were centrifuged at 4500 rpm (Rotanta/TR, Hettich, Tuttlingen, Germany) for 10 min at 4 °C. The supernatant was transferred and directly analysed. Supernatants of CYP1A2 (phenacetin to paracetamol) and CYP2E1 (chlorzoxazone to 6-hydroxychlorzoxazone) marker reactions were diluted with 10 mmol/l potassium phosphate, pH 3.2 in a 1:1 ratio prior to analysis.

IC_{50} values were determined by non-linear least-square regression analysis using GraFit version 3.0 (Erithacus Software Limited, Middlesex, UK), Fig. 5.

2.4. Analytical system

An Agilent 1100 system (Waldbronn, Germany) consisting of an autosampler, a degasser, a binary pump, and a variable-wavelength detector was used. An FP920 fluorescence detector (Jasco, Tokyo, Japan) was coupled in-line with the UV detector. A 100×4.6 mm Chromolith Performance (Merck) chromatographic column was used for analytical separation employing 10 mmol/l potassium phosphate buffer at pH 3.2, alternatively, pH 7.2, as solvent A1, methanol as solvent B1, and acetonitrile as solvent B2. Buffer reservoir A2 contained deionised water and was used for automatic rinsing of the system from phosphate buffer after running a sample batch. The analytical column was protected with a 0.5 μm stainless steel inline filter (Upchurch Scientific, Oak Harbor, WA, USA). The analytes were separated isocratically within 2 min at mobile phase compositions summarised in Table 1. These conditions were determined in standard solutions containing 33% methanol to mimic sample composition. Following isocratic separation, the column was rinsed of remaining substrate as well as hydrophobic

Table 1
Chromatographic and detection method conditions for the metabolites of the cytochrome P450 marker reactions

CYP isoform	Substrate	K_m* (μmol/l)	Metabolite	λ (nm)	Eluent	Alternative eluent	Flow-rate (ml/min)	k'
1A2	Phenacetin	20	Paracetamol	244	Phosphate, pH 3.2–MeOH (85:15)	–	3	0.92
2A6	Coumarin	3	7-OH-Coumarin	324 ex., 454 em.	Phosphate, pH 3.2–ACN (78:22)		5	1.04
						Phosphate, pH 3.2–MeOH (63:37)	5	1.06
2C8	Paclitaxel	8	6α-OH-Paclitaxel	231	Phosphate, pH 3.2–ACN (51:49)		3	1.14
						Phosphate, pH 3.2–MeOH (32:68)	3	1.07
2C9	Diclofenac	5	4-OH-Diclofenac	267	Phosphate, pH 3.2–MeOH (38:62)		3	1.31
				280		Phosphate, pH 3.2–ACN (55:45)	3	1.04
2C19	S-Mephenytoin	20	4-OH-Mephenytoin	224	Phosphate, pH 3.2–ACN (79:21)		3	1.04
						Phosphate, pH 3.2–MeOH (65:35)	3	1.09
2D6	Bufuralol	20	1-OH-Bufuralol	247 ex., 297 em.	Phosphate, pH 3.2–ACN (83:17)		5	1.08
						Phosphate, pH 3.2–MeOH (67:33)	5	1.11
2E1	Chlorzoxazone	100	6-OH-Chlorzoxazone	297	Phosphate, pH 3.2–ACN (85:15)		3	1.53
						Phosphate, pH 3.2–MeOH (71:29)	3	1.38
3A4	Midazolam	4	1-OH-Midazolam	240	Phosphate, pH 7.2–MeOH (40:60)		3	1.46
						Phosphate, pH 7.2–ACN (60:40)	3	1.36
3A4	Testosteron	70	6β-OH-Testosteron	254	Phosphate, pH 3.2–MeOH (50:50)	–	3	1.35

*K_m = Michaelis–Menten constant, determined in the laboratory [22].

Table 2
Time scheme for the chromatographic run

Time (min)	Event
0	Injection
0–2	Isocratic separation and data acquisition
2–2.2	Gradient to 90% B1 or B2
2.2–3	Column wash at 90% B1 or B2
3–4.5	Column equilibration

matrix components and equilibrated, see Table 2. Detection wavelengths for the various analytes were determined in the respective mobile phase and are summarised in Table 1. Instrument control and data acquisition were performed with Chromeleon (Dionex, Germering, Germany).

Calibration curves were constructed by linear regression from three analyses of standards at six concentration levels and a blank sample. Analyses were performed with the eluents described in column 6 of Table 1. Standards were prepared by spiking HLM incubations lacking substrate (blank matrix). Precision was determined by analysing control samples in replicates of eight at three concentrations on four separate occasions. For accuracy determination, agreement between measured and nominal concentration of the control samples were assessed.

3. Results and discussion

The monolithic porous silica column allows high speed separation in combination with good separation. This provides an excellent tool to modernise LC–UV and LC–fluorescence routines in analytical support of in vitro cytochrome P450 assays. For the cytochrome P450 isoforms involved in most biotransformation of therapeutics, suitable marker reactions have been identified and are chosen for their enzyme specificity [23], examples are listed in Fig. 1. In principle, the marker reactions can be monitored by (A) a decrease in substrate or (B) an increase in metabolite concentration. The occurrence of the metabolite is more specific than the decrease of substrate, therefore, method development focused on metabolite analysis.

As detection of several of the analytes occurs with limited selectively, namely by UV absorbance at low wavelengths, and sample pretreatment is minimal, efficient chromatographic separation is important in order to minimize matrix interferences. In order to obtain satisfactory separation efficiencies, a 100 mm column was chosen rather than the Chromolith Speedrod (50 mm). In addition, the metabolites were separated isocratically. In comparison to previously published LC–UV and LC–fluorescence methods with typical run times in the range of 20 min [4–6], separation on the monolithic column reduces overall sample run time to 4.5 min. In this way, solvent consumption per sample is in the same order of magnitude despite increased flow-rates.

3.1. Chromatographic conditions

For fast chromatographic run times, short retention times are necessary. Nevertheless, sufficient retention for separation from the matrix front is required, for which reason a capacity factor of 1–2 was aimed for. This was achieved for all analytes with exception of paracetamol, see Table 1. Retention of paracetamol can be increased by reducing methanol content in samples and eluent, e.g., a capacity factor of 1.71 was achieved for fully aqueous samples employing a mobile phase comprising 10% methanol. Nevertheless, the lower capacity factor for paracetamol in methanol-containing samples was accepted to minimise deviations from the sample preparation procedure.

From a practical point of view acetonitrile is preferred over methanol as organic solvent as it causes less backpressure. For most analytes, both acetonitrile and methanol are suitable as mobile phase constituents and can thus be chosen freely. The exceptions are paracetamol, where acetonitrile-based mobile phases have too strong an elution power, and 6β-hydroxytestosteron, which shows improved peak shape when employing methanol-based mobile phases. When real life samples contain NCEs which interfere with the analyte peak, a switch between acetonitrile and methanol may provide a convenient key to successful analysis.

Mobile phase additives typical in earlier methods were avoided in order to accommodate analysis for all marker reactions with minimal set-up changes. A low pH phosphate buffer proved satisfactory for all analytes but 1-hydroxymidazolam. At low pH midazolam is positively charged at the imidazole

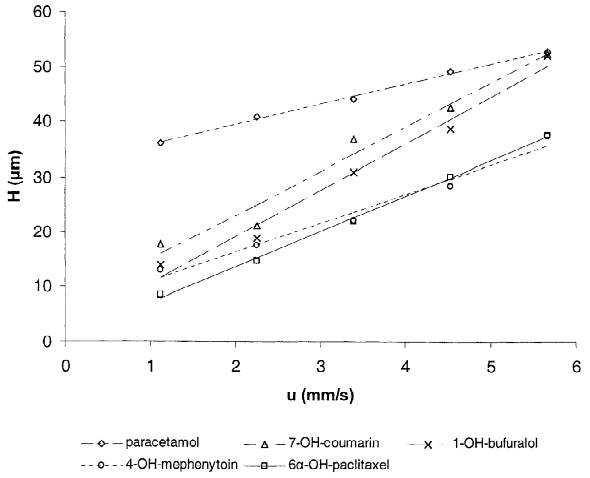


Fig. 2. Van Deemter plots for paracetamol, 7-hydroxycoumarin, 6α-hydroxypaclitaxel, 1-hydroxybufuralol, and 4-hydroxy-mephenytoin. Mobile phases (column 6) and detection conditions as compiled in Table 1, for further details, see Experimental.

nitrogen, entailing similar retention behaviour as its metabolite. By using a buffer with pH 7.2, which is above the pK_a of midazolam (theoretical value 5.65), separation is also achieved for this marker reaction.

In Fig. 2, the Van Deemter curves are plotted for a number of the analytes. Although not dramatic, plate heights are shown to increase with increased linear velocity. To obtain chromatographic run times below 5 min without compromising separation efficiency, a flow-rate of 3 ml/min was chosen unless detection occurs by means of fluorescence. As the fluorescent analytes 7-hydroxycoumarin and 1-hydroxybufuralol are detected more selectively, reduced separation efficiency at increased flow-rate is acceptable for further speeding up analysis. Here, chromatographic run times for the fluorescent analytes was reduced to 2.7 min.

Sensitivities obtained and linear ranges investigated are listed in Table 3 and are comparable to earlier laboratory methods [4]. Data for precision and accuracy for 1-hydroxymidazolam as example was acceptable as shown in Table 4.

3.2. Sample treatment

In comparison to other biological matrices, microsomal incubations are relatively clean samples, in principal containing microsomal proteins, salts, and

Table 3
Sensitivity and linearity data for metabolite analysis of the cytochrome P450 marker reactions

CYP isoform	Metabolite	LOD* (μmol/l)	Linear range investigated (μmol/l)	Slope	SD in slope	Intercept	SD in intercept	Standard error
1A2	Paracetamol	0.05	0.1–10	0.0549	0.00017	−0.000106	0.000603	0.00250
2A6	7-OH-Coumarin	0.004	0.05–10	16.6	0.03	0.473	0.0801	0.403
2C8	6α-OH-Paclitaxel	0.01	0.02–2	0.312	0.0010	−0.00243	0.000796	0.00313
2C9	4-OH-Diclofenac	0.03	0.04–7	0.213	0.0011	−0.0122	0.00265	0.0133
2C19	4-OH-Mephenytoin	0.03	0.06–10	0.0853	0.00042	−0.00178	0.001483	0.00732
2D6	1-OH-Bufuralol	0.001	0.025–3	37.6	0.06	−0.0357	0.07490	0.3584
2E1	6-OH-Chlorzoxazone	0.06	0.1–10	0.0348	0.00055	0.00151	0.002146	0.00874
3A4	1-OH-Midazolam	0.03	0.1–20	0.243	0.0002	0.00738	0.001485	0.00748
3A4	6β-OH-Testosteron	0.08	0.2–20	0.124	0.0002	0.00278	0.001180	0.00596

For details, see Experimental. *LOD=Limit of detection determined as $S/N=3$.

Table 4
Precision and accuracy for the determination of 1-hydroxymidazolam in HLM incubations

CYP isoform	Metabolite	Nominal concentration (μmol/l)	Overall precision (RSD, %)	Accuracy (%)
3A4	1-OH-Midazolam	0.127	7.71	109.9
		0.670	3.61	96.5
		6.89	2.06	92.5

For details, see Experimental.

NADPH. For compatibility with analytical flow systems, the microsomal proteins need to be removed which can be achieved with the same means as stopping the enzymatic reaction, i.e., by means of precipitation. As shown in Fig. 3, employing a denaturing agent such as methanol that has a relatively low molar absorptivity in comparison to, e.g., formic acid, is favourable to reducing background interference. This is especially important for separations relying on low capacity factors. For six of the marker reactions, methanol precipitation proved adequate. For analysis of 4-hydroxydiclofenac, samples require acidification to achieve adequate separation of 4-hydroxydiclofenac from the front as the 4-hydroxydiclofenac carboxy functionality (theoretical pK_a 4.17) is charged at the neutral pH of the assay conditions. Separation of paracetamol and 6-hydroxychlorzoxazone on the other hand is affected by the elution strength of methanol precipitated samples, which therefore are diluted prior to analysis.

3.3. Ruggedness

System ruggedness is not only dependent on chromatographic conditions, but also on practical routines. Therefore, analytical procedures for all marker reactions were kept as uniform as possible with minimum set-up alterations. A single LC system was used containing methods for instrument control, including analytical conditions and automatic system washing, as well as methods for integration and report definitions, thus allowing a convenient walk-up analytical system. The choice of hardware was based on instrumentation currently available in the metabolism laboratory. By employing LC equipment capable of flow-rates above 5 ml/min and exchanging detector flow cells, linear velocities, and thus throughput, can possibly be further increased.

3.4. Application

The system described in this paper is currently being implemented in the authors' laboratory to support metabolism research. Example chromatograms of real life samples are shown in Fig. 4. So far, interference from matrix components or NCEs added to the assay have not posed a problem.

Fig. 5 presents results on evaluating the inhibitory effects of test compounds on cytochrome P450 3A4 catalysed testosteron 6β-hydroxylation using human liver microsomes. From these curves, the inhibitory concentration which results in 50% reduction in enzyme activity (IC_{50}) is determined. Each assay run is controlled by a selective inhibitor. Results of test

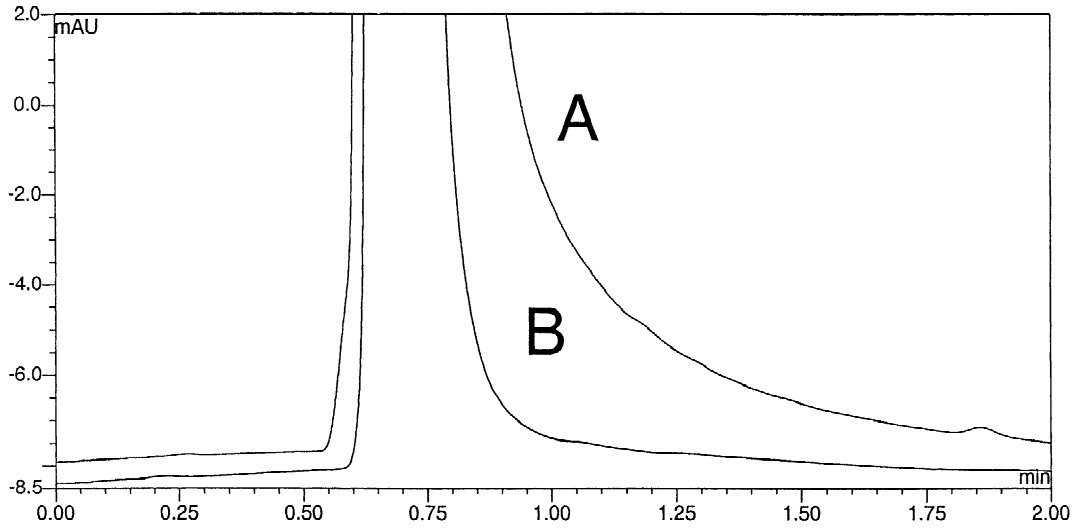


Fig. 3. Blank incubation mixture precipitated with (A) 100 μl 2% (v/v) formic acid, and (B) 100 μl methanol. Experimental conditions as for 6β-hydroxytestosteron, see Table 1.

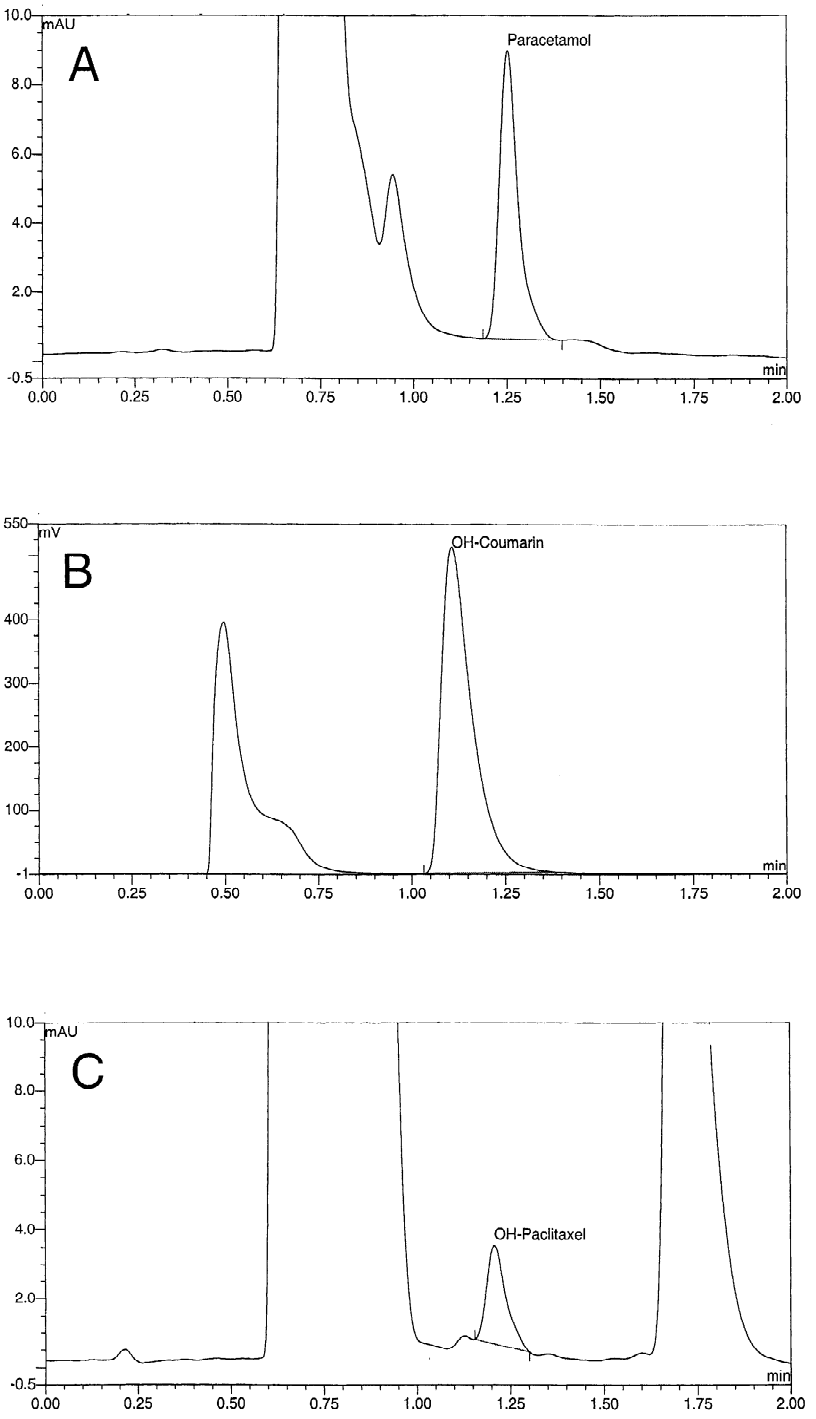


Fig. 4. Chromatograms of real life samples for (A) paracetamol, (B) 7-hydroxycoumarin, (C) 6α-hydroxypaclitaxel, (D) 4-hydroxydiclofenac, (E) 4-hydroxymephenytoin, (F) 1-hydroxybufuralol, (G) 6-hydroxychlorzoxazone, (H) 1-hydroxymidazolam, and (I) 6β-hydroxytestosteron. Mobile phases (column 6) and detection conditions as compiled in Table 1, for further details, see Experimental.

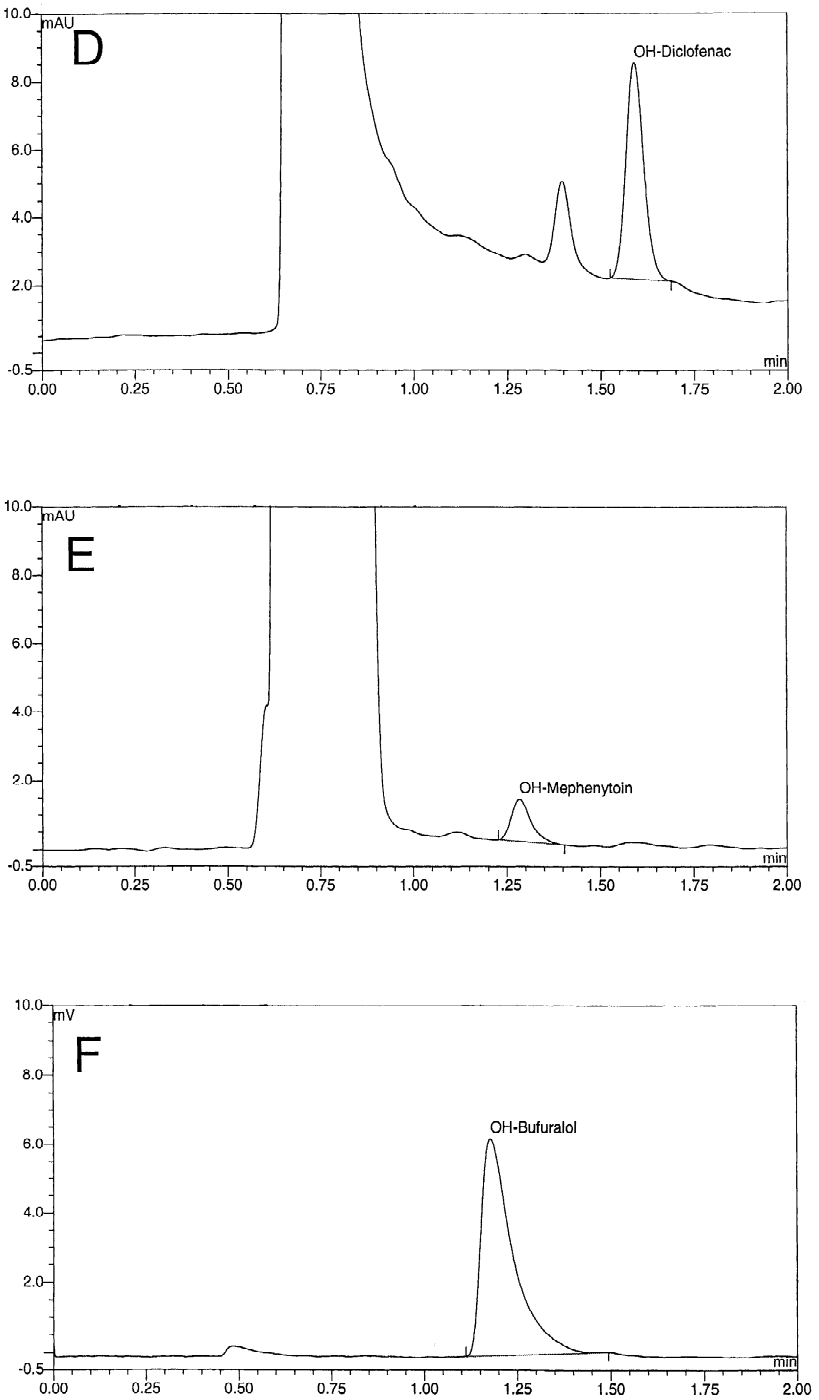
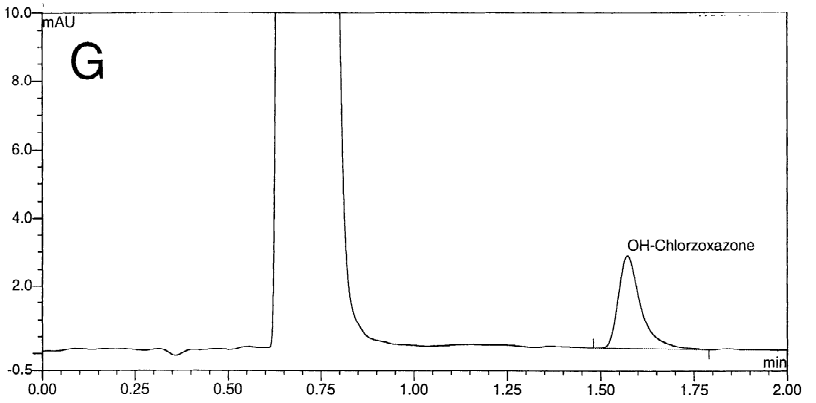
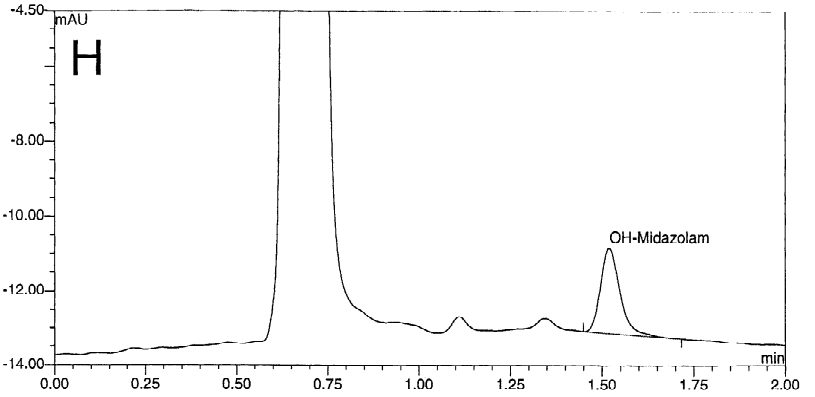


Fig. 4. (*continued*)





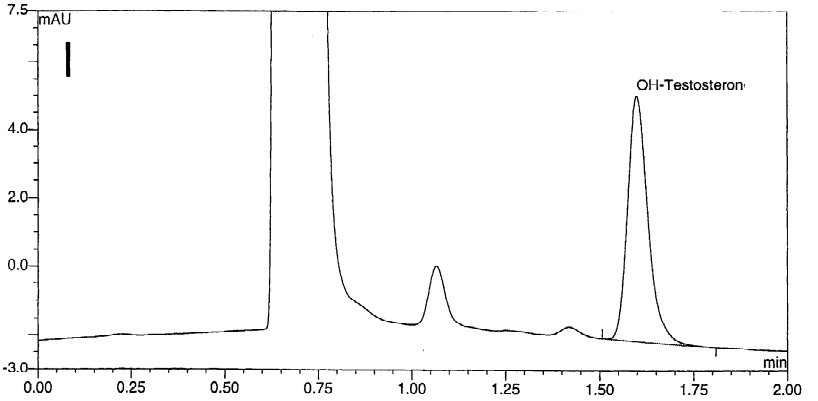


Fig. 4. (*continued*)

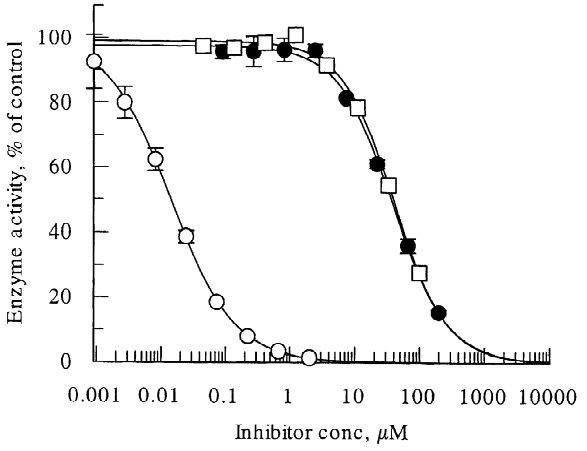


Fig. 5. Evaluation of the inhibitory effects of test compounds and ketoconazole on CYP3A4 catalysed testosteron 6β-hydroxylation using human liver microsomes. For assay conditions, see Experimental.

compound runs are only considered valid, when the positive control inhibitor satisfies a set range of IC_{50} value. The positive control inhibitors used in our laboratory are: α-naphthoflavone for 1A2, tranylcypromine for CYP2A6, quercetin for CYP2C8, sulfaphenazole for CYP2C9, ticlopidine for CYP2C19, quinidine for CYP2D6, and ketoconazole for CYP3A4. In Fig. 5, ketoconazole gave an IC_{50} of 0.014 μmol/l which is within the expected range.

4. Conclusions and perspectives

Monolithic silica-based chromatography was successfully applied to cytochrome P450 marker reaction analysis. In comparison to previously reported methods, simplified analytical procedures are presented providing sufficient chromatographic resolving power in a reduced run time maintaining sensitivity and linearity. Sample throughput per day is thus increased significantly and can, in principle, be doubled again when taking advantage of the column wash and equilibration time, e.g., by means of a parallel column set-up. Availability of appropriate precolumn material will further increase robustness, and reduced column diameters are of interest for improving assay sensitivity and reducing solvent consumption. The approach described in this paper has proven to be applicable to diverse classes of compounds, it is thus expected that this approach is easily adapted to monitor other cytochrome P450 marker reactions that previously have been analysed by LC–UV and LC–fluorescence.

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