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European Journal of Pharmaceutical Sciences 12 (2000) 151–158

EUROPEAN JOURNAL OF PHARMACEUTICAL SCIENCES

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Evaluation of a novel high-throughput assay for cytochrome P450 2D6 using 7-methoxy-4-(aminomethyl)-coumarin

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Received 6 March 2000; received in revised form 26 April 2000; accepted 26 April 2000

Dedicated to Professor D.D. Breimer on the occasion of the 25th anniversary of his appointment to the Chair of Pharmacology at Leiden University.

Abstract

We recently reported on the design, synthesis and characterisation of a novel and selective substrate of human cytochrome P450 2D6 (CYP2D6), 7-methoxy-4-(aminomethyl)-coumarin (MAMC). Here, we describe a high-throughput microplate reader assay, which makes use of MAMC as a fluorescent probe for determining the inhibition and activity of CYP2D6 in heterologously expressed systems and human liver microsomes. The high-throughput screening (HTS) assay can be used both in an end-point and real-time configuration, and is easy to use, rapid and sensitive. In addition, end-point measurements by means of flow injection analysis have also successfully been performed. The HTS-assay was validated by performing inhibition experiments for several low- and high-affinity ligands ($n=6$) of CYP2D6, and comparing the findings to those obtained with the standard *O*-demethylation assay of dextromethorphan. The results indicate that all compounds tested display competitive inhibition in both the MAMC and dextromethorphan assay, and the K_i values reveal a very good correlation ($R^2=0.984$) between the two assays. To further demonstrate the usefulness of the HTS-assay, IC_{50} values of a series of five N-substituted analogs of 3,4-methylenedioxyamphetamine for CYP2D6 have been determined. The results obtained demonstrate that the current HTS-assay represents a significant improvement over previous assays, with a higher turnover of MAMC and a higher selectivity for CYP2D6.

Keywords: Human CYP2D6; High-throughput screening; Microplate reader; Flow injection analysis; Inhibition; Validation

1. Introduction

The application of combinatorial chemistry in the discovery and development of new drug candidates has led to an increasing demand for high-throughput screening methods that are capable of identifying enzymes involved in the binding (prediction of drug–drug interactions) and, potentially, the metabolism of new ligands (Rodrigues, 1997). As hepatic cytochromes P450 (CYPs) are the enzymes mainly responsible for the metabolism of therapeutic agents, several efforts have been made to develop such methods for individual CYPs (Crespi et al., 1997). Next to human CYP3A4 and CYP1A2, one of the isoenzymes of particular interest in this regard is human cytochrome P450 2D6 (CYP2D6, EC 1.14.14.1) (Spatzenegger and Jaeger, 1995; Tanaka and Breimer, 1997). This is not only due to its important role in drug metabolism, but also because it is deficient with varying frequencies in different populations, as a result of a variety of genetic defects (Kroemer and Eichelbaum, 1995; Wormhoudt et al., 1999). This so-called debrisoquine/sparteine polymorphism (Eichelbaum et al., 1975; Mahgoub et al., 1977) can lead to a reduced bioactivation capacity towards prodrugs, as well as undesired clinical responses to drugs, due to high plasma levels and altered metabolic pathways of a therapeutic agent (Breimer, 1983; Tucker, 1994). High affinity binding to CYP2D6 may, therefore, be used as a negative selection criterion in the process of drug discovery and development.

Recently, we have reported on the design and synthesis of a novel and selective substrate of CYP2D6, 7-methoxy-4-(aminomethyl)-coumarin (MAMC) (Onderwater et al.,

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0928-0987/00/$ – see front matter
PII: S0928-0987(00)00150-0

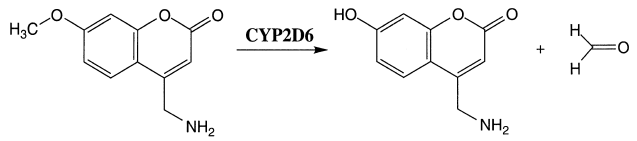

Fig. 1. *O*-Demethylation of 7-methoxy-4-(aminomethyl)-coumarin (MAMC) to its 7-hydroxyl derivative HAMC, mediated by CYP2D6.

1999). Characterisation of this compound revealed that MAMC was selectively *O*-demethylated to 7-hydroxy-4-(aminomethyl)-coumarin (HAMC; Fig. 1) by CYP2D6, with only CYP1A2 contributing to the formation of this metabolite to a small extent in human liver microsomes. The latter contribution could, however, be eliminated completely by the addition of the highly selective CYP1A2 inhibitor furafylline. As the fluorescent properties of MAMC and its metabolite HAMC were found to be significantly different, it was suggested that MAMC could be an interesting tool for high-throughput screening.

Until recently, only classical assays based on HPLC and GC techniques have been described for inhibition of CYP2D6 using substrates such as dextromethorphan, bufuralol and metoprolol (Kronbach et al., 1987; Lennard and Silas, 1983). At this moment two high-throughput assays have been described (Crespi et al., 1997; Rodrigues et al., 1994). However, the substrate used in the first assay, 7-ethoxy-3-cyano-coumarin, is not selective for CYP2D6 and has a much lower turnover than MAMC (Onderwater et al., 1999). The second assay has the disadvantage that it makes use of radioactively labelled dextromethorphan.

In this study, we evaluated a high-throughput microplate reader assay using MAMC as a novel fluorescent probe for determining IC_{50} or K_i values. The method was validated by comparing the inhibition constants (K_i values) obtained with the high-throughput assay, with those from the frequently used *O*-demethylation assay of dextromethorphan. Both specific and non-specific ligands of CYP2D6, displaying a wide range of binding affinities, were used as a test-set. In addition, kinetic studies on MAMC *O*-demethylation were performed in human liver microsomes to investigate the applicability of the microplate reader assay to this system. To further demonstrate the usefulness of the high-throughput assay, IC_{50} values were determined for a series of *N*-substituted analogs of 3,4-methylenedioxyamphetamine (MDA). These compounds are of interest because CYP2D6 has been implicated in the neurotoxic effect of the *N*-methyl and *N*-ethyl derivatives of MDA (MDMA and MDE, respectively) (Ensslin et al., 1996; Lin et al., 1997).

2. Materials and methods

2.1. Chemicals

MAMC and HAMC were synthesised as described previously (Onderwater et al., 1999). Structural analogs of 3,4-methylenedioxyamphetamine were synthesised according to Shulgin (1991). Glucose-6-phosphate dehydrogenase was from Boehringer (Mannheim, Germany). NADPH was obtained from Applichem (Darmstadt, Germany). SK&F 525A was from SmithKline&French Laboratories (Herts, UK). All other chemicals were obtained from Sigma (St Louis, MO, USA).

2.2. Microsomal protein

Human cytochrome P450 2D6 (CYP2D6) microsomal protein expressed in a human lymphoblastoid cell line (catalogue no. P171) was purchased from GENTEST (Woburn, MA, USA). The CYP2D6 content was 175 pmol/mg microsomal protein. Human liver microsomes of a single individual were a generous gift from Dr. B. Rademaker (Rephartox, The Netherlands).

2.3. Calibration curve of HAMC

Calibration curves of HAMC were made for quantification purposes. To 100 μl of a mixture containing the appropriate amount of microsomal protein and different concentrations of HAMC (0.625–40 μM final concentration) in a 100 mM phosphate buffer (pH 7.4), 100 μl of a preincubated NADPH-regenerating system (see below) were added. Subsequently, 10 μl 5 M trichloroacetic acid (TCA) were added. The pH was readjusted to pH 9 by the addition of 100 μl of a 1.5 M Tris–HCl buffer (pH 9.0). The fluorescence of HAMC was measured at excitation and emission wavelengths of 405 nm (bandwidth 8 nm) and 460 nm (bandwidth 30 nm), respectively, on a Victor^2 1420 multilabel counter (Wallac, Turku, Finland).

2.4. Time and protein dependency of HAMC formation

Prior to the inhibition studies, linearity of the formation of HAMC and its protein dependency were investigated by incubating 40 μM MAMC at 37°C in a 100 mM phosphate buffer (pH 7.4) containing 20, 40 or 60 nM CYP2D6, and a NADPH-regenerating system containing a final concentration of 0.1 mM $NADP^+$ (see below). The amount of HAMC formed was assayed as described below at different time-points.

2.5. Assay of MAMC O-demethylation for IC_{50} measurements

IC_{50} measurements were performed in costar 3595 96-well plates using a 100 mM phosphate buffer (pH 7.4) and a final volume of 200 μl. Briefly, a concentration range of inhibitor was obtained by serially diluting 50 μl of a solution containing the appropriate amount of inhibitor, with 100 μl of buffer solution. Subsequently, 50 μl of a

mixture containing 40 nM CYP2D6 and 40 μM MAMC were added. After 10 min of preincubation at 37°C, 50 μl of a preincubated cofactor solution was added to start the reaction, resulting in final concentrations of 0.1 mM NADPH, 0.3 mM glucose-6-phosphate, 0.4 mM $MgCl_2$, and 0.4 units/ml glucose-6-phosphate dehydrogenase. After 45 min, the reaction was stopped by adding 10 μl of a 5 M TCA solution. Prior to analysis, the pH was adjusted with 100 μl of a 1.5 M Tris–HCl buffer (pH 9.0). Quantification of HAMC formation was performed using the standard curve described above.

2.6. Assay of MAMC O-demethylation for K_i measurements

The *O*-demethylation of MAMC was investigated in costar 3595 96-well plates, in both the absence and presence of two different concentrations of inhibitor, to establish apparent K_m and V_{max} values. The incubations were performed in a 100 mM phosphate buffer (pH 7.4) with a final volume of 200 μl. A concentration range of MAMC (1.25–160 μM final concentration) was obtained by serially diluting 100 μl of substrate solution with 100 μl of buffer. Subsequently, 25 μl of a solution containing the appropriate amount of inhibitor (determined by IC_{50} measurements), and 25 μl of a solution containing CYP2D6 (10 nM final concentration) were added. After a preincubation (37°C) of 10 min, 50 μl of a preincubated solution (37°C) containing a NADPH-regenerating system were added (see above). After 45 min the reaction was stopped by the addition of 10 μl of a 5 M TCA solution. Prior to analysis, 100 μl of a 1.5 M Tris–HCl buffer (pH 9.0) was added to the incubation mixture in order to increase the fluorescent signal of HAMC. The amount of HAMC formed was quantified by measuring the fluorescence at excitation and emission wavelengths of 405 (bandwidth 8 nm) and 460 nm (bandwidth 30 nm), respectively, on a Victor^2 1420 multilabel counter (Wallac).

2.7. Flow injection analysis of HAMC formation

As an alternative to measuring HAMC formation with a microplate reader, a flow injection analysis (FIA) setup was used. The incubations were basically performed as described above. However, after the addition of TCA, the precipitated protein was first centrifuged for 15 min at 4000×*g*, and 100 μl of 1.5 M Tris–HCl buffer (pH 9.0) were added to 180 μl of supernatant. HAMC formation was measured by injecting 100 μl of sample into a HPLC apparatus, consisting of a model 305 pump (Gilson Medical Electronics, WI, USA); a Waters 717 plus autosampler (Waters, MA, USA) cooled to 4°C; and a model 821-FP fluorescence detector (Separations, The Netherlands). As eluent 50:50 (v/v) methanol:H_2O was used at a flow rate of 1 ml/min. Detection was at an excitation wavelength of 405 nm (bandwidth 18 nm) and an emission wavelength of 480 nm (bandwidth 18 nm), with an analysis time of 1.0 min. For quantification, a calibration curve of HAMC was used.

2.8. MAMC O-demethylation in human liver microsomes

In addition to heterologously expressed CYP2D6, the applicability of the microplate assay was also investigated in human liver microsomes. The incubations were generally carried out as described above; however, the microsomal fraction and NADPH-regenerating system were pre-incubated for 5 min at 37°C in the presence of 30 μM furafylline (Onderwater et al., 1999), before starting the reaction by the addition of substrate. Also, microsomal protein was removed from the stopped incubation mixtures by centrifugation, and the supernatant transferred into a 96-well plate for analysis by microplate reader as described above. Protein-dependency of HAMC formation was investigated in human liver microsomes by incubating 40 μM of MAMC with varying amounts of microsomal protein (0.12–0.36 mg/ml). Samples were drawn after 0, 15, 30, and 45 min to determine the linearity of the reaction. Additional incubations, also containing 1 μM quinidine, were performed to ensure that the observed HAMC formation was solely CYP2D6 mediated. Enzyme kinetics of MAMC *O*-demethylation were studied using 0.30 mg/ml microsomal protein.

2.9. Assay of dextromethorphan O-demethylation

The *O*-demethylation of dextromethorphan to its metabolite dextrorphan was investigated both in the presence and absence of two different concentrations of inhibitor, in a 100 mM phosphate buffer (pH 7.4). A mixture containing dextromethorphan (2.5–40 μM final concentration), CYP2D6 (8.3 nM final concentration), and the appropriate amount of inhibitor (as determined by IC_{50} measurements), in a total volume of 100 μl, was pre-incubated for 5 min at 37°C. The reaction was started by the addition of 100 μl of a 2 mM NADPH solution, in a 100 mM phosphate buffer (pH 7.4). After 10 min the reaction was stopped by the addition of 100 μl 3 M perchloric acid. The incubation mixture was subsequently centrifuged for 15 min at 4000×*g*, and the supernatant was analysed by HPLC.

2.10. HPLC analysis of dextrorphan

Chromatographic separation of dextromethorphan and its metabolite dextrorphan was achieved with a HPLC system consisting of a model 305 pump (Gilson Medical Electronics); a Waters 717 plus autosampler (Waters); a Suplex pKb-100 column (4.6 mm×15 cm) and corresponding guard column (Supelco, PA, USA); and a model 821-FP fluorescence detector (Separations). The mobile phase consisted of 25% acetonitrile, 1% triethylamine, and

74% H_2O, pH 3.0, delivered at a flow rate of 1 ml/min. The injection volume was 100 μl. Dextrorphan was detected at excitation and emission wavelengths of 280 and 311 nm (bandwidth 18 nm), respectively. For quantification, a calibration curve of dextrorphan was used (0.1–10 μM). The analysis time was 20 min.

2.11. Kinetic analyses

Apparent K_m and V_{max} parameters were determined by using the non-linear least-square fitting method for one site binding, as implemented in Prism 2.0 (GraphPad Software, USA). K_i values were subsequently calculated with the following equation:

$$K_i = (K_m/(K'_m - K_m)) \cdot [I]$$

In the equation, K_m is the apparent K_m value in the absence of inhibitor, K'_m is the apparent K_m value in the presence of inhibitor, and [I] is the inhibitor concentration. IC_{50} values were determined by using the non-linear least-square fitting method for one site competition, as implemented in Prism 2.0 (GraphPad Software).

3. Results

3.1. Linearity of HAMC formation by heterologously expressed CYP2D6

We have investigated the time- and protein-dependent formation of HAMC from MAMC, at a substrate concentration of 40 μM. As can be seen in Fig. 2a, the formation of HAMC by CYP2D6 was linear in time, for at least 45 min, at all P450-concentrations investigated. Metabolite formation was also found to be linear with respect to protein concentration (Fig. 2b).

3.2. Enzyme kinetics of heterologously expressed CYP2D6

The metabolism of MAMC to its *O*-demethylated metabolite HAMC by CYP2D6 displayed apparent Michaelis–Menten kinetics, as demonstrated by Fig. 3. Apparent K_m and V_{max} values measured by the microplate assay on different days were 7.9±2 μM and 8.1±0.7 min^{-1} ($n=7$, Table 1), respectively. The detection limit for HAMC quantification by the microplate reader was 0.3 μM (signal-to-noise ratio, 3:1). Kinetic studies performed on separate days using the FIA method resulted in similar K_m and V_{max} values of 6.4±0.6 μM and 7.3±0.2 min^{-1} ($n=6$, Table 1), respectively. This method displayed a detection limit of 0.1 μM for HAMC.

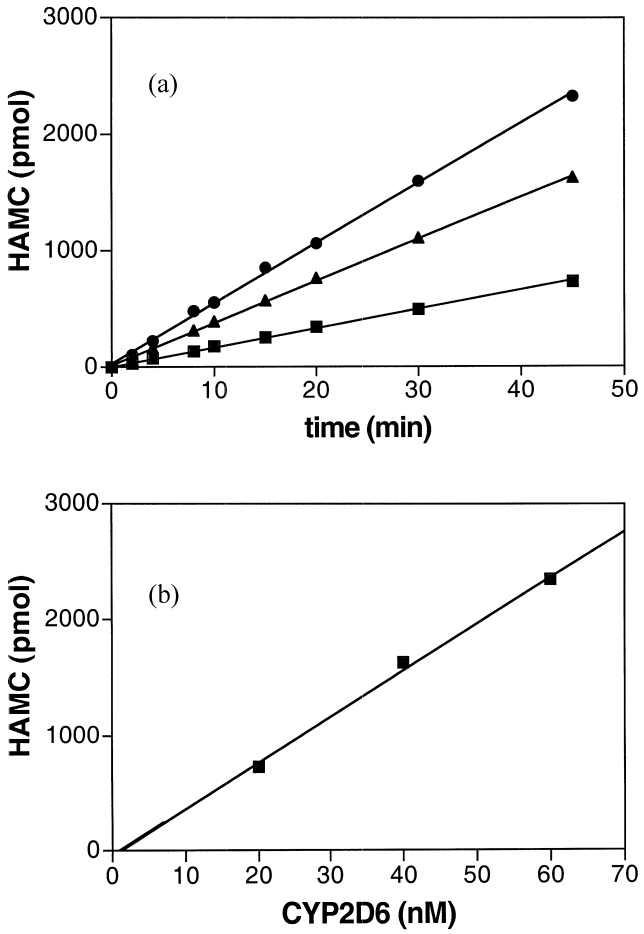

Fig. 2. (a) Time-dependent formation of HAMC from MAMC, in the presence of 20 (■), 40 (▲) and 60 (●) nM CYP2D6. Incubations were performed at 37°C in a 100 mM phosphate buffer (pH 7.4). (b) Protein-dependency of MAMC *O*-demethylation to HAMC by CYP2D6, shown for an incubation of 45 min at 37°C in a 100 mM phosphate buffer (pH 7.4).

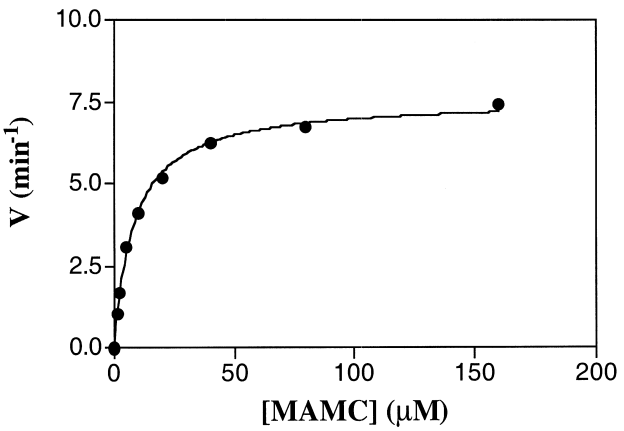

Fig. 3. Rate of *O*-demethylation of MAMC to its 7-hydroxyl metabolite HAMC by CYP2D6 plotted against the substrate concentration. Incubations were performed in a 100 mM phosphate buffer (pH 7.4).

Table 1
Apparent K_m and V_{max} values for, respectively, the *O*-demethylation of MAMC and dextromethorphan by CYP2D6, expressed in a human lymphoblastoid cell line ($n=6$)[a]

Substrate	Assay	K_m (μM)	V_{max} (min^{-1})
MAMC	Microplate	7.9±2.0	8.1±0.7
MAMC (human liver)	Microplate	34.6±2.0	251±14[b]
MAMC	FIA	6.4±0.6	7.3±0.2
Dextromethorphan	HPLC	2.0±0.6	4.8±0.3

[a] The enzymatic parameters for MAMC *O*-demethylation were additionally determined in human liver microsomes from a single individual ($n=3$). Values are the mean±S.D.
[b] In pmol/min per mg microsomal protein.

3.3. MAMC O-demethylation in human liver microsomes

HAMC formation in human liver microsomes, pre-incubated for 5 min with 30 μM furafylline, displayed linearity for at least 45 min. MAMC *O*-demethylation was also linear with varying quantities of microsomal protein. In the presence of both 30 μM furafylline, a selective CYP1A2 inhibitor, and 1 μM quinidine, a selective CYP2D6 inhibitor, no HAMC formation could be detected. The metabolism of MAMC in the human liver microsomes displayed apparent Michaelis–Menten kinetics. Apparent K_m and V_{max} values were found to be 34.6±2.0 μM and 251±14 pmol/min per mg microsomal protein, respectively.

3.4. Inhibition studies

Inhibition of the CYP2D6 mediated *O*-demethylation of dextromethorphan ($K_m=2.0\pm0.6$ μM, $n=6$) by MAMC, showed that MAMC is a relatively potent competitive inhibitor of this model substrate of CYP2D6, with a K_i value of 10.8±3.1 μM ($n=3$, Fig. 4). Additionally, dextromethorphan was found to be a competitive inhibitor of HAMC formation, with a K_i value of 0.66±0.15 μM ($n=3$).

Table 2
Apparent K_i values for the inhibition of MAMC *O*-demethylation by different ligands of human lymphoblast-expressed CYP2D6[a]

Ligand	K_i value (μM)
Quinidine	0.0029±0.0004
SK&F 525A	0.044±0.015
Dextromethorphan	0.66±0.15
Triprolidine	1.0±0.42
Sparteine	16.1±5.6
Codeine	24.0±3.4

[a] Values are the mean±S.D. ($n=3$).

To further validate the microplate assay for MAMC *O*-demethylation, K_i values were measured for a number of known ligands of CYP2D6, with both the dextromethorphan and the MAMC *O*-demethylation assay (Table 2). For this purpose a selection of specific and non-specific ligands of CYP2D6 was made, displaying a wide range of inhibitory potencies. The specific ligands included codeine, sparteine, and quinidine, whereas the non-specific ligands were triprolidine and SK&F 525A. All ligands of CYP2D6 studied displayed competitive inhibition towards both dextromethorphan and MAMC. As shown in Fig. 5, a good correlation of $R^2=0.984$ was obtained between the K_i values from both assays.

IC_{50} values for CYP2D6 of a series of N-substituted analogs of 3,4-methylenedioxyamphetamine (MDA) were determined. The measurements were performed at a final concentration of MAMC close to the K_m value (10 μM). As can be seen in Table 3, elongation of the *N*-alkyl chain initially resulted in an increased inhibitory potency of the ligand for CYP2D6. This trend is, however, discontinued when the *N*-propyl moiety is replaced by a *N*-butyl group.

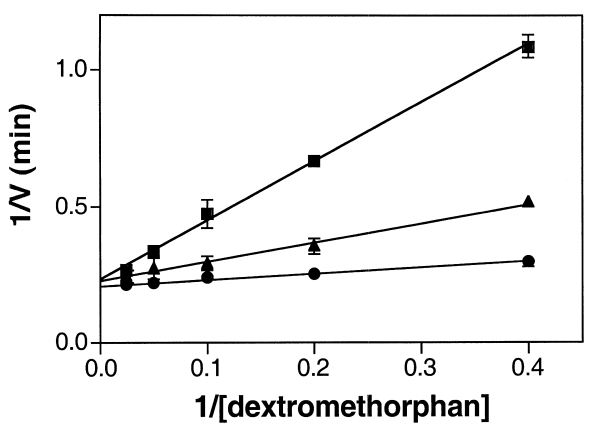

Fig. 4. Lineweaver–Burke plot for dextromethorphan *O*-demethylation by CYP2D6, in the presence of 0 μM (●), 40 μM (▲) and 80 μM (■) MAMC. Incubations were performed in a 100 mM phosphate buffer (pH 7.4).

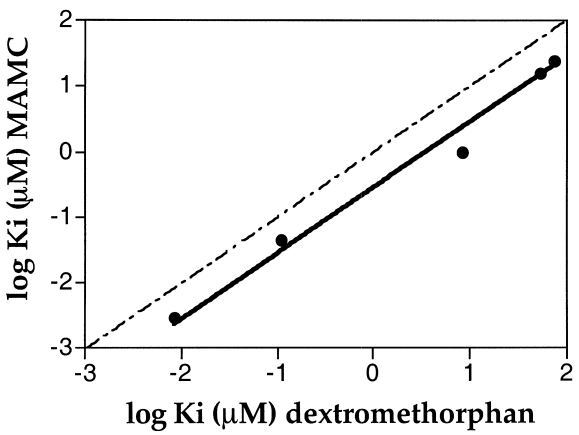

Fig. 5. Plot of the apparent K_i values obtained with the dextromethorphan *O*-demethylation assay versus those obtained with the MAMC *O*-demethylation assay. A correlation of $R^2=0.984$ was obtained. The trend in inhibitory potency is: quinidine>SK&F 525A>triprolidine>sparteine>codeine.

Table 3
IC_{50} values for the inhibition of the CYP2D6 mediated *O*-demethylation of MAMC by a series of N-substituted MDA analogs[a]

MDA analog	IC_{50} value (μM)
R = Methyl	0.92±0.14
R = Ethyl	0.44±0.06
R = Propyl	0.096±0.014
R = Butyl	0.19±0.03
R = Phenethyl	190±70

[a] CYP2D6 was expressed in human lymphoblasts. Values are shown as the mean±S.D. (n = 3).

The *N*-phenethyl substituted analog displayed the lowest capacity for inhibiting the enzyme.

4. Discussion

The aim of this study was to evaluate a novel and selective high-throughput assay for measuring inhibition of CYP2D6, heterologously expressed and in human liver microsomes, in order to identify potential ligands of CYP2D6. MAMC was used as the substrate, because this was recently demonstrated to be a selective substrate of CYP2D6 potentially suitable for high-throughput screening, due to the fluorescent properties of its metabolite HAMC (Onderwater et al., 1999).

Initial kinetic studies on the *O*-demethylation of MAMC by CYP2D6 demonstrated that a good signal-to-background ratio could be obtained with a protein concentration as low as 10 nM CYP2D6 per well, when an incubation time of 45 min was used. In order to minimise the amount of CYP2D6 required, this concentration and incubation time were used for the inhibition experiments. As noted previously, NADPH interferes with the fluorescent signal of HAMC (Onderwater et al., 1999). This interference could, however, be reduced satisfactorily to background levels by using a final concentration of 0.1 mM $NADP^+$ in the NADPH-regenerating system, and an excitation wavelength of 405 nm. The stop-solution used was a 5 M TCA solution, as it was found that the addition of 100 μl acetonitrile–0.1 M Tris, pH 9.0 (60:40, v/v), the stop-solution used in other high-throughput assays (Crespi et al., 1997), interferes with our assay. To increase the fluorescent yield of HAMC, 100 μl of a Tris–HCl buffer, pH 9.0, was added. It should be noted that when the samples were not analysed immediately after addition of this base, the incubation mixtures had to be stored in the dark at 4°C.

Although we have described an end-point measurement of HAMC formation, we have also performed some real-time measurements, during which the increase in fluorescence was measured every 5 min. An advantage of the latter method is the fact that the level of the background signal is less disturbing, as activity is determined by the slope of the increase in fluorescence. Because the slope can be determined more easily, the incubation time can be reduced. Also, the enzymatic reaction does not have to be stopped. However, to make the assay suitable for use with a multi-plate reader, we used an end-point measurement of HAMC formation.

We also examined the use of flow injection analysis (FIA) as an alternative to quantification of HAMC by a microplate reader. The FIA method was found to require only some small adaptations of the microplate assay and represents a significant improvement in analysis time compared to a classical HPLC method, since no chromatographic separation is required. An advantage of FIA over a microplate reader is the reduction in background signal, due to the fact that protein has been removed from the incubation mixture. This results in a higher sensitivity, as reflected by the lower detection limit for HAMC. However, it also makes the FIA assay more laborious. It should be noted that, in the case of the FIA assay, an emission wavelength of 480 nm was used, as this was optimal for reducing the background level (Onderwater et al., 1999).

The incubations containing human liver microsomes clearly show the advantages of MAMC over other fluorescent probes for CYP2D6. The P450 selectivity of MAMC is such that, in the presence of the specific CYP1A2 inhibitor furafylline, HAMC formation is solely attributable to CYP2D6. This is demonstrated by the fact that addition of quinidine, combined with furafylline, completely inhibited MAMC metabolism. Measurements of MAMC *O*-demethylation using heterologously expressed CYP2D6 in the presence and absence of 30 μM furafylline demonstrated that the latter is a weak inhibitor of CYP2D6 at this concentration, resulting in a factor 1.5 increase in K_m value. The addition of furafylline alone, however, cannot explain the increase in K_m value observed in human liver microsomes. Instead, the higher K_m value obtained in human liver microsomes may additionally be due to (aspecific) binding of MAMC to other proteins and/or a relatively high amount of CYP2D6. Alternatively, as only one human liver sample was investigated it cannot be excluded that the microsomes contained a polymorphic form of CYP2D6 which differs from the heterologously expressed CYP2D6, resulting in different binding characteristics. The difference in K_m value does not, however, affect the ability of the assay to identify new ligands of CYP2D6, which could potentially cause drug–drug interactions. When compared to heterologously expressed CYP2D6, only small modifications to the microplate reader assay are required in order to obtain a very rapid method for screening the CYP2D6 activity in human liver microsomes.

The present inhibition studies show that MAMC is a competitive inhibitor of dextromethorphan, and vice versa, which is in agreement with previous molecular modelling results (Onderwater et al., 1999; De Groot et al., 1997; Koymans et al., 1992). Comparison of the K_i values obtained for the series of low- and high-affinity ligands

with both the MAMC and dextromethorphan assay, furthermore, revealed a very good correlation ($R^2 = 0.984$, Fig. 5) between the two methods. It can also be seen that the K_i values obtained with the two assays differ in absolute value, the K_i values from the MAMC *O*-demethylation assay (Table 2) being consistently smaller. Importantly, this difference constitutes a constant factor. The observed trend in a 4-order range of inhibitory potency of the studied ligands is, thus, identical for the two methods. Theoretically, K_i values should be independent of the substrate used, provided the binding of the different substrates is of a truly competitive nature. This ideal situation is, however, difficult to accomplish experimentally, since no two CYP2D6 substrates, belonging to different chemical classes, are likely to share the exact same interaction points within the active site of the enzyme. It is therefore not surprising that significant differences in K_i values generally arise between methods that use different substrates (Tucker, 1998). The fact that the K_i values for MAMC are smaller than those for the larger substrate, dextromethorphan, can be rationalised by the ability of the latter to form more lipophilic interaction points with active site residues, making its displacement more difficult. Another explanation may be the use of slightly different incubation conditions. It can, therefore, be concluded that MAMC *O*-demethylation, measured by the present microplate assay, is a valid tool for determining binding affinities of ligands of CYP2D6.

In order to illustrate the potential use of inhibition of MAMC *O*-demethylation for large-scale screening purposes, we have determined IC_{50} values for a series of *N*-substituted analogs of 3,4-methylenedioxyamphetamine (MDA) with the microplate reader. As can been seen from Table 3, IC_{50} values were determined reproducibly. The microplate assay thus also provides an adequate screening tool for identifying structural analogs, which display a lower potential for drug–drug interactions. In the case of the MDA analogs, substitution at the basic nitrogen with a phenethyl-group resulted in the highest IC_{50} value for CYP2D6 and, by inference, the lowest risk of drug–drug interactions.

The present results with MAMC clearly show that this compound is an excellent high-throughput probe for determining ligand affinities for CYP2D6. Its advantages compared to classical assays, e.g., the previously described dextromethorphan *O*-demethylation assay, mainly reside in its speed. The MAMC *O*-demethylation assay is also much less laborious. This is due to the fact that it is not necessary to remove microsomal protein from the incubation mixtures, and because serial dilutions can be made. Furthermore, the assay is sensitive and requires only small amounts of CYP2D6. As an alternative to quantifying HAMC by microplate reader, FIA can be used. Another very important feature of MAMC as a probe is its selectivity, making it highly suitable for screening of CYP2D6 activity in human liver microsomes. The present MAMC *O*-demethylation assay can be viewed as a significant improvement over the existing 7-ethoxy-3-cyanocoumarin *O*-deethylation assay ($K_m = 67$ μM, $V_{max} = 0.017$ min^{-1}; Crespi et al., 1997) as MAMC is a much more selective substrate of CYP2D6, with a more than 400-fold higher turnover. Recently, GENTEST introduced 3-[2-(*N*,*N*-diethyl-*N*-methylamino)ethyl]-7-methoxy-4-methylcoumarin (AMMC) as a new CYP2D6 substrate, suitable for high-throughput screening (Crespi, 1999). However, the fluorescent characteristics of AMMC and its metabolite, and the turnover number of AMMC by CYP2D6 ($V_{max} = 1$ min^{-1}), are inferior to those of MAMC. The use of AMMC thus either requires a higher concentration of CYP2D6, or a lower concentration of NADPH in order to decrease background fluorescence.

In conclusion, a novel high-throughput microplate reader assay is available for determining the inhibition and activity of CYP2D6 in heterologously expressed systems and human liver microsomes, which is easy to use, rapid and sensitive. The assay makes use of the selective CYP2D6 substrate 7-methoxy-4-(aminomethyl)-coumarin (MAMC) as a fluorescent probe, and represents a significant improvement over previous assays, with a higher selectivity for CYP2D6 and higher turnover of MAMC. In addition to the microplate reader-based assay, flow injection analysis can also be used. In the case of human liver microsomes, pre-incubation with furafylline fully eliminates the contribution to HAMC formation by CYP1A2, which is the only P450 slightly active towards MAMC besides CYP2D6.

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