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Metabolism of Mometasone Furoate and Biological Activity of the Metabolites.

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ABSTRACT

In order to better evaluate the pharmacokinetic and pharmacodynamic properties of the new inhaled glucocorticoid mometasone furoate (MF), the metabolism of MF was evaluated in rat and human tissues and in rat after iv administration. Metabolic studies with ^{3}H-MF in human and rat plasma and S9 fractions of human and rat lung showed relatively high stability, and a degradation pattern similar to that seen in buffer systems. MF was efficiently metabolized into at least five metabolites in S9 fractions of both rat and human liver. There were, however, quantitative differences in the metabolites between the two species. The apparent half-life of MF in the S9 fraction of human liver was found to be 3 times greater compared to that in rat. MET1, the most polar metabolite, was the major metabolite in rat liver fractions where as both MET1 and MET2 were formed to an equal extent in human liver. Metabolism and distribution studies in rats after intravenous and intratracheal administration of [1,2-^{3}H]-MF revealed that most of the radioactivity (~90%) was present in the stomach, intestines and the intestinal contents, suggesting biliary excretion of MF and its metabolites. Radiochromatography showed that most radioactivity was associated with MET1, MET2 and MET 3. Fractionation of the HPLC-eluate (MET1-5) revealed that only MF (RBA 2900) and MET2 (RBA 700) had appreciable glucocorticoid receptor binding affinity. These results suggest that MF undergoes distinct extrahepatic metabolism, but generates active metabolites that might be in part responsible for the systemic side effects of MF.

Mometasone furoate (MF, Scheme 1) is a potent glucocorticoid that has been used in the treatment of topical dermatological disorders and allergic rhinitis (Onrust and Lamb, 1998; Prakash and Benfield, 1998). Recently, MF has been approved in Europe and the United States as an oral inhalation powder, in the treatment of mild-to-moderate persistent asthma (Bernstein et al., 1999; Affrime et al., 2000a; Nayak et al., 2000; Sharpe and Jarvis, 2001). There is very limited information on the pharmacokinetics of MF after an oral inhalation. Affrime et al. reported the PK after a single dose administration of 400 µg of MF given intravenously, by a metered dose inhaler (MDI) and by a dry powder inhaler (DPI) (Affrime et al., 2000a). Following the administration by MDI, MF was undetectable in plasma and after the DPI administration, plasma concentrations of MF were also extremely low and close to the limit of quantification (LOQ, 50 pg/mL). The mean bioavailability of MF after DPI was estimated to be 0.96% (Affrime et al., 2000a). Minimal systemic exposure to MF was also seen after multiple-dose administration by DPI with mean C_{max} values close to or below the LOQ (50 pg/mL) after a 200 to 800 µg/day dose administered by DPI for 4 to 52 weeks (Bernstein et al., 1999; Affrime et al., 2000b). These initial studies on the PK of MF suggest that the systemic levels and bioavailability of MF are extremely low after an oral inhalation when compared to other inhaled glucocorticoids. The reason for these observations might be either that MF exhibits a more efficient systemic clearance via extrahepatic metabolism or through low pulmonary availability. Recent studies (Derks, 2005)have shown that the systemic availability of MF might not be as low as suggested previously. Also, the drug shows considerable systemic effects as manifested by the suppression of the hypothalamic-pituitary-adrenal axis (Fardon et al., 2004), although original reports claimed minimal cortisol suppression (Affrime et al., 2000b). A further complication is that the formation of active MF metabolites (Isogai et al., 1993) might be responsible in part for

the observed systemic effects despite the low levels reported by Affrime. Because of these controversies, we were interested in further evaluating pharmacokinetic and pharmacodynamic properties of MF, which included hepatic and extrahepatic metabolism, and the assessment of the bioactivity of formed metabolites.

The information available on the hepatic metabolism of MF is limited. An abstract reported that MF undergoes extensive metabolism *in vitro* by liver hepatocytes (Zbaida et al., 1997). Other studies have suggested that MF undergoes hydroxylation at the 6-position to form 6β-hydroxy-MF, hydrolysis of the furoate ester and the substitution of the C-21 chlorine with a hydroxyl group (Onrust and Lamb, 1998; Affrime et al., 2000a; Crim et al., 2001). The above-mentioned suggested routes of metabolism have not been confirmed and there is no evidence of a single major metabolite (Sharpe and Jarvis, 2001). Two reports, by Teng et al., on the metabolism of unlabeled MF in liver and intestinal microsomes reported the formation of one metabolite (tentatively identified as 6β-hydroxy-MF) while mentioning that other parallel and subsequent metabolic pathways could be involved (Teng et al., 2003a; Teng et al., 2003b). These studies also reported MF to be stable in plasma and lung tissue. In order to be able to capture the overall metabolism of MF in a more complete fashion we decided to evaluate the hepatic and extrahepatic metabolism of MF *in-vitro* as well as *in-vivo*, using a radiolabed tracer and to assess the biological activity of the whole metabolic spectrum rather then performing such studies for just one single metabolite. These studies should be able to confirm whether extrahepatic metabolism might explain the low systemic levels after inhalation of MF, give information about the generated metabolites and their pharmacological activity and information on the biodistribution and elimination of the drug.

Results of these studies would be able to support or argue against the pharmacokinetic reports by Affrime (Affrime et al., 2000b). Distinct extrahepatic metabolism would support Affrimes pharmacokinetic findings of low systemic MF levels and reduced systemic side effects (in the absence of active metabolites) (Affrime et al., 2000b). Assuming extrahepatic metabolism, the existence of active metabolites might explain the reported cortisol suppression (Fardon et al., 2004) despite low MF levels. In the second case, the lack of extrahepatic metabolism and the formation of only minor amounts of active metabolites would argue against the reported pharmacokinetic profile of MF and the possibility that MF does not differ from other inhaled glucocorticoids with respect to safety issues.

MATERIALS AND METHODS

Chemicals

Mometasone furoate (MF) was purchased from USP (Rockville, MD, USA) and [1,2-^{3}H]-mometasone furoate (specific activity: 0.56 Mbeq/mmole) was provided by AstraZeneca (Lund, Sweden). All other chemicals and solvents were obtained from Sigma Chemicals Co. (St Louis, MO) and Fisher Scientific Co. (Cincinnati, OH).

The use of Sprague Dawley rats (Harlan, Indianapolis, IN) was approved by the local Institutional Animal Care and Use Committee (IACUC) at the University of Florida, Gainesville, Florida. Approval was obtained from the Institutional Review Board, University of Florida for the use of human lung and liver tissues.

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Stability of MF in plasma

The stability of MF in fresh rat plasma was assessed by incubating the plasma spiked with MF, at a concentration of 6.2 µM, at 37°C in a thermostatically controlled water bath. Experiments were conducted in triplicates. 200 µl aliquots were removed at regular intervals up to 72 h into pre-chilled tubes and the samples were precipitated with 600 µl of methanol. Blank rat plasma was used in a control experiment. Identical stability studies were performed using fresh human plasma also. The samples were stored at –20°C and were analyzed by HPLC within 48 h of storage.

The precipitated plasma samples were vortex mixed and centrifuged at 10000 rpm for 5 min and 100 µl of the supernatant was injected directly for HPLC-UV analysis. A LDC/Milton Roy CM4000 multiple solvent delivery system using a Milton Roy SM 4000 programmable wavelength detector set at λ_{max} 254 nm, a CR-3A Chromatopac integrator (Shimadzu Corporation, Japan) and an automatic injector (Perkin Elmer, Boston, MA) was used as the HPLC system. Chromatographic separation of the analytes was achieved on Waters 5-µm symmetry RP-18 (150 x 4.6 mm i.d) column (Milford, MA) preceded with a guard column (10 x 4.6 mm i.d, filled with reverse phase pre-column material) using a mobile phase of 65:35% v/v methanol-water at a flow rate of 1 ml.

Calibration curves based on MF peak areas over the concentration range of 0.125 to 5.0 µg/mL using unweighted least squares linear regression analysis resulted in r^2 values of at least 0.999. The limit of quantification of the assay was 0.125 µg/ml. The good reproducibility of the system

allowed quantification without an internal standard as potential internal standards cross-eluted with either the endogenous interferences or the degradation products formed.

Because of the lack of suitable standards for the degradation products, quantification was based on the assumption that their molar absorptivities were identical to that of MF. Results for MF and degradation products are presented in µM and expressed in % of MF starting concentrations in the graphical representations.

Metabolism of MF in liver and lung

Preparation of homogenizing buffer (1.15% KCl in 50 mM K_2HPO_4 pH 7.4)

870 mg of dipotassium hydrogen orthophosphate (K_2HPO_4) and 1.15 g of potassium chloride (KCl) was dissolved in 100 ml of deionized water and the pH was adjusted with 20% v/v orthophosphoric acid to 7.4. The buffer was chilled to 4°C before use.

Preparation of NADPH generating system

NADPH generating system, which comprised of cofactors salts: NADP (2 mM), Glucose-6-phosphate (8 mM), Nicotinamide (200 mM) and Magnesium chloride (200 mM) was prepared in the homogenizing buffer. This cofactor solution was incubated at 37°C for 15 min to allow generation of NADPH and then was used immediately.

Tissue metabolism

S9 fractions of rat and human lung and liver were used for studying the metabolism of MF. Human tissue samples were obtained from the Molecular Tissue Bank at the University of

Florida. These samples were obtained by the tissue bank from surgical procedures (liver and lung resection, non-smokers) but further information was not obtainable from the Molecular tissue bank. Samples were stored at -70^{o} C. Rat tissues were obtained by sacrificing the rats by decapitation and removing the lung and liver tissue immediately. The tissues were then rinsed in ice-cold homogenizing buffer, blotted dry and weighed.

The lung and liver tissue were homogenized in ice-cold homogenizing buffer to obtain 20 and 40% w/v of tissue respectively. The lung and liver tissue homogenate were centrifuged at 4°C at 10000x g for 45 min to obtain the S-9 fraction for lung and liver. S9 fractions were pre-incubated with equal volumes of cofactor solution and incubated in a shaker bath to equilibrate the mixture to 37°C. Unlabeled MF was added to initiate the reaction and the final drug concentration was 4.8µM and final tissue concentration was 10% w/v for lung and 20% w/v for liver. The lung and liver S9 fractions were incubated at 37°C for up to 24 h under atmospheric air. Drug free S9 fraction (lung or liver) was used in a control experiment. Serial samples of 250µl were withdrawn at 0, 5, 10, 15, 20, 25, 30, 40, 50, 60, 90, 120, 240 min and 24 h into pre-chilled tubes. The samples were precipitated with 250µL of acetonitrile and analyzed by HPLC. The precipitated samples of lung and liver homogenates were centrifuged at 10000 rpm for 5 min. 100 µl of the supernatant was injected directly HPLC analysis. The chromatographic conditions for analysis of samples from S9 studies was identical to those mentioned earlier for analysis of the plasma samples. However, a mobile phase composition of 50:50% v/v of acetonitrile-water was used for the analysis of the S9 samples.

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Results for MF and metabolites are presented in µM and expressed in % of MF starting concentrations in graphical representations. Semi logarithmic plots of % MF remaining versus time were used to determine the apparent disappearance rate constant of MF. The disappearance half-life was determined using the equation $t_{1/2} = 0.693/k_{diss}$, where k_{diss} is the apparent disappearance rate constant for that compound.

Metabolism in liver and lung tissue using labeled [1,2-^{3}H]-MF

An identical parallel incubation was performed simultaneously with 5 ml lung and liver S9 fraction in which [1,2-^{3}H]-MF (concentration <0.125 µg/ml) was added in addition to the unlabeled MF (2.5 µg/ml). The homogenizing buffer was also spiked with identical concentrations of labeled and unlabelled MF and was used in a control experiment. The samples containing labeled MF were also incubated as mentioned in the previous section. 250 µl aliquots were withdrawn at every 20 min for the first two hours and finally at 24 h. The samples were withdrawn into pre-chilled tubes and were precipitated with 250 µl of acetonitrile to terminate the reaction. The samples were immediately analyzed using HPLC using the conditions mentioned for the analysis of unlabeled MF. 1 min fractions were collected using a fraction collector and transferred to individual scintillation vials and counted using a liquid scintillation counter (Beckman Instruments LS 5000 TD, Palo Alto, CA) for the presence of radioactivity. The total counts present for MF at time zero were considered as 100%. The percentages of metabolites formed were estimated as the ratio of dpm for metabolite to dpm of parent drug at time zero.

In vivo metabolism and distribution of MF

A total of 5 µCi of [1,2-^{3}H]MF per 250 mg of bodyweight, in normal saline was administered by injection into the tail vein of male Sprague-Dawley rats. The animals were sacrificed by decapitation after two hours. The abdominal and thoracic cavities were opened immediately and blood from the heart was withdrawn. Urine was collected by puncturing the urinary bladder. The organs (Table 1) were dissected, rinsed with ice-cold normal saline, blotted, weighed and then homogenized in methanol. 1 cm^2 skin and the vastus lateralis muscle from the right leg were resected and homogenized without being rinsed. The contents of the stomach and the intestines (both small and large) were flushed out of the organs before homogenization and collected. 30 ml of methanol was used to homogenize the liver and 20 ml was used to homogenize the other tissues. 1 ml of the homogenates was transferred to scintillation vials and 10 ml of the scintillation cocktail was added. The scintillation vials were read in a liquid scintillation counter (Beckman Instruments LS 5000 TD, Palo Alto, CA).

Glucocorticoid receptor binding assay experiments in rat lung cytosol

As described earlier in the metabolism experiments, liver S9 fractions were spiked only with unlabeled MF and incubated at 37°C in a shaker bath for 2 h. The 2 h samples were analyzed by HPLC-UV analysis and the metabolites were isolated by collecting the fractions corresponding to the retention times of the metabolites. The fraction corresponding to the elution of MF (denoted by MF_{frac}) in the samples was also collected. These fractions were then evaporated under vacuum and reconstituted in methanol. The concentrations of the MF_{frac} and the metabolites in the fractions were calculated from % conversion of MF into the metabolites data obtained from the previous section. The dilutions for MF (from a stock of 1 mg/mL), MF_{frac} and

the metabolites were prepared in methanol. Dexamethasone (DEXA) was used as a reference compound for the receptor binding experiments. The concentration ranges for the compounds in the study were 0.01 to 100 nM for MF, MET1 and MET3; 0.01 to 30 nM for MF_{frac} and MET2; 0.01 to 50 nM for MET4 and MET5 and 0.01 to 1000 nM for DEXA.

A previously described method (Hochhaus and Moellmann, 1990), with slight modifications, was used for performing the competition assays. Sprague Dawley rats were anesthetized using a cocktail mixture of ketamine, xylazine and acepromazine (3:3:1 v/v) and were decapitated. The lungs were removed and homogenized in 8 volumes of ice-cold incubation buffer (10 mM Tris/HCL, 10 mM sodium molybdate, 2 mM 1,4-dithioerythritol). The homogenate was incubated with 5% w/v charcoal suspension (in deionized water) for 10 min. The homogenate was then centrifuged for 20 min at 40,000X *g* in J2 rotor of Beckman centrifuge to obtain the cytosol. Fresh cytosol was prepared and used for all the individual experiments.

A final tracer concentration of 10 nM of ^{3}H-labeled dexamethasone solution was used as a tracer, based on previous saturation binding experiments performed in our laboratory (data not shown). 10 µl of the dilutions of the test compound in methanol was added to pre-chilled tubes. Blank methanol was used for the determination of total binding. Non-specific binding was determined after addition of 10 µl of 100 µM unlabeled DEXA (10 µM in the final incubation mixture). 10 µl of 100 nM ^{3}H-dexamethsone solution (10 nM in final incubation mixture) was then added. 80 µl of the lung cytosol was added and the tubes were vortexed and incubated at 4°C for 24 h. After the incubation, 100 µl of 5% w/v charcoal suspension (in water) was added to the tubes to remove the excess unbound radioactivity. The tubes were vortexed and 150 µl of the supernatant

was transferred to the scintillation vials. 5 ml of the scintillation cocktail was added and the scintillation vials were read in the liquid scintillation counter. Control experiments were also conducted in which receptor binding assays were performed on fractions collected during HPLC-UV analysis of blank S9 fractions and on fractions collected during blank mobile phase runs.

The data obtained were fitted by SCIENTIST^{TM} (Micromath, Salt Lake City, UT) using the following E_{max} model to obtain the estimates of B_{max} and IC_{50}.

$$DPM = B_{max} - B_{max} \frac{C^N}{IC_{50}{}^N + C^N} + NS$$

where DPM represents the total tracer binding obtained at any given competitor concentration, NS represents non-specific binding and N the Hill coefficient. B_{max} is the specific binding by the ligand in the absence of competitor.

The IC_{50} obtained for dexamethasone ($IC_{50,dex}$) was used to calculate the relative binding affinity (RBA_{test}) of the test compound form its IC_{50} value ($IC_{50,test}$) as:

$$RBA_{test} = \frac{IC_{50,dex}}{IC_{50,test}} \times 100$$

RESULTS

Stability in plasma

The stability of MF was studied in fresh plasma harvested from both rat and human blood. Incubation of MF in rat plasma at 37°C for 72 h led to the formation of three degradation products D1, D2 and D3. However, MF was found to be stable in plasma until 6 h of incubation.

The formation of degradation products D1, D2 and D3 occurred after 20 h of incubation. Using mass spectrometry (positive electro-spray ionization) method reported elsewhere (Sahasranaman et al., 2004), the molecular masses ([MH^+]) of MF, D1, D2 and D3 were determined to be 521, 485, 467 and 503 respectively. The polarity sequence of MF and its degradation products were in the order of D1>MF>D2>D3. The decline and formation of MF and D1, D2 and D3 is shown in Figure 1(a). The degradation half-life of MF as calculated from the terminal phase of the concentration-time curve was found to be 85 h.

MF was also found to be stable in human plasma until 6 h of incubation at 37°C. However, prolonged incubation of MF in human plasma for 72 h led to the formation of identical degradation products (D1, D2 and D3, Figure 1(b)) as observed in rat plasma. The degradation half-life calculated for MF in human plasma was found to be 24 h which was 3.5 times shorter compared to that in rat plasma.

Stability in lung

Incubation of MF in rat lung S9 fractions showed no metabolic conversion until 2 h. However, incubation of MF for 24 h resulted in a degradation product, D2 that was found to be more non-polar than the parent drug. This degradation product had identical retention times as the product D2 seen upon incubation of MF in plasma. Parallel control incubations of labeled MF in the incubation buffer also showed formation of D2. This indicated that the formation of D2 was a result of break down of MF in the homogenizing buffer and not a result of metabolism in the lung. Incubations of labeled MF in the S9 fraction of lung were also conducted, which also proved that MF was stable in lung fractions. The representative radiochemical elution profiles for

MF in rat lung incubations are shown in Figure 2. Incubation of MF in human lung S9 fractions also indicated that MF was stable with no metabolic conversion until 2 h of incubation. These results indicate that MF does not undergo significant pulmonary metabolism.

Metabolism of MF in liver

Incubation of a mixture of non-labeled and labeled MF with rat liver S9 fractions over 1 h showed formation of five metabolites (Figure 3). The retention times for the metabolites MET1, MET2, MET3, MET4 and MET5 were between 1-2, 5-6, 7-8, 11-12 and 12-13 min respectively. A semi logarithmic plot of % dpm versus time displayed a linear relationship with a correlation coefficient of 0.956 indicating first order elimination kinetics for MF. The disappearance half-life of labeled MF was 18.5 min in rat liver S9 fractions. The concentration-time profiles of MF and its metabolites are shown in Figure 4. At the end of 1 h, MET1 was observed as the major conversion product with MET/MF ratio of 32%. Seven percent of MF was converted to MET2. The sum of the MET/MF ratios for metabolites 3, 4 and 5 was less than 10%. Only about 30% of the total radioactivity was found to correspond to MF during 1 h of incubation. The remaining radioactivity corresponded to background noise. The control buffer sample did not show any formation of polar degradation products indicating that the formation of metabolites was a result of enzymatic activity present in the S9 fraction of liver sample and not a result of degradation in the buffer.

The HPLC-UV profiles observed after incubation of unlabeled-MF with S9 fraction of rat liver indicated the formation of a polar metabolite (MET2) that eluted at 5.1 min. The disappearance half-life of unlabelled MF (16 min) as determined by HPLC-UV analysis was found to be

identical to the half-life of labeled MF (18.5 min) that was estimated from the radiochemical elution profiles. The metabolite MET1 (retention time between 2-3 min) could not be viewed using a UV-VIS detector due to the presence of endogenous interferences at the same retention time. MET3, MET4 and MET5 could not be quantified using UV due to the very low concentrations of these metabolites.

Incubation of a mixture of labeled and unlabelled MF in human liver S9 fractions also showed formation of five metabolites. The retention times for these metabolites were identical to the ones formed with rat liver S9 fractions. A semi logarithmic plot of % dpm versus time displayed a linear relationship with a correlation coefficient of 0.9584 and the disappearance half-life of ^{3}H-MF in the S9 fraction was found to be 0.76 h^{-1} with a corresponding half-life of 55 min. The concentration-time profile for the disappearance of MF and appearance the metabolites are shown in Figure 5. The HPLC-UV profiles observed after incubation of MF with S9 fraction of human liver showed an endogenous tissue impurity eluting at around 5 min. Because of this interference, MET2 could not be observed in human liver tissues. Hence, only the decline of MF could be monitored under UV detection over time. The molecular mass ([MH^{+}]) of MET2 was estimated to be 538 using a mass spectrometry method reported previously (Sahasranaman et al., 2004).

In vivo metabolism and disposition

The radioactivity (expressed as % of dose administered) in the tissues 2 h after an intravenous injection of [1,2-^{3}H]-MF in rats is given in Table 1. Radiochemical elution profiles of the intestinal contents show the formation of three metabolites that have identical retention times as

the metabolites MET1, MET2 and MET3 (Figure 6), which were seen upon incubation of MF with liver S9 fractions. There was no radioactivity corresponding to MF and this is consistent with the efficient hepatic metabolism of MF that was seen *in vitro*. Most of the radioactivity in the intestinal contents was associated with MET1 (Figure 6) while MET2 and MET3 were minor metabolites. MET4 and MET5 were either not formed or formed in too low a concentration to be seen in the intestinal contents.

Glucocorticoid receptor binding of MF and its metabolites

The relative binding affinities of DEXA, MF and the isolated metabolites were determined in a competition binding experiment in rat lung cytosol. Table 2 gives the relative binding affinities (RBA) values for DEXA, MF, MF_{frac} and the metabolites. The competition curves for the compounds are shown in Figure 7. In all binding experiments, non-linear curve fitting revealed that slope factors (Hill coefficient) for all the compounds were close to unity. Minute fractions collected during HPLC-UV analysis of blank S9 fractions and during blank mobile phase runs did not show any binding to the glucocorticoid receptor.

DISCUSSION

The first part of study investigated the stability and metabolism of MF in lung and plasma in order to answer the question as to whether or not MF is cleared by extrahepatic metabolism. MF was relatively stable in rat and human plasma. This high stability in plasma and lung tissue agreed with findings of Teng et al. (Teng et al., 2003a; Teng et al., 2003b). Our results, however,

propose a different degradation pathway (Scheme 1) which was based on more detailed investigations of D1 and D2 in buffer systems. The structure of D3 has been proposed based on the structures of D1 and D2 and the molecular mass of D3. There is no conclusive evidence in this study for the conversion of D3 to D2 therefore this pathway has been denoted by broken arrows in the scheme. D1, but not D2 (Sahasranaman et al., 2004) and presumably not D3 (considering similarity to D2) show binding to the glucocorticoid receptor.

Even though degradation of MF is observed in plasma and lung *in vitro*, the relative high stability, when compared to the hepatic processes, makes it unlikely that extrahepatic metabolism is responsible for the low systemic exposure of MF after an oral inhalation. Other events, such as the removal of the lipophilic, slow dissolving drug from the lung through mucociliary clearance might be, in part, responsible for low systemic levels. Very recently, the systemic bioavailability of mometasone furoate has been shown to differ between subjects with normal and reduced lung function (Mortimer et al., 2005), an indication for the removal of the more centrally deposited drug in patients with lower lung function. Contrary to this, there is also some indication that the systemic exposure after MF inhalation is actually higher than originally reported (Derks, 2005). Therefore, it seems likely that systemic availability of MF is not being determined by extrahepatic clearance, but can be explained solely on the pharmacokinetic fate of MF in the lung.

In human plasma, the sum of MF and degradation products at 72 hr did not add up to 100%. Hoegger suggested covalent binding of the epoxide(s) (Valotis et al., 2004) which would prevent extraction**.** In that paper 9-16% covalent binding was found after 3 hours of incubation. This binding, presumably to protein components, explains also the reduced extractable fraction found

in our experiments, and therefore supports Hoegger's finding. However, future studies are necessary to show whether such adducts would also be formed *in vivo*.

This study showed that MF is efficiently metabolized into at least five metabolites in both rat and human liver S9 fractions. There were, however, quantitative differences in the metabolites between the two species. The apparent half-life of MF in the S9 fraction of human liver was found to be 3 times greater compared to that in rat. MET1, the most polar metabolite, was the major metabolite in rat liver fractions whereas both MET1 and MET2 were formed to an equal extent in human liver fractions. Such species differences have been noted for other glucocorticoids such as budesonide(Edsbacker et al., 1987).

The molecular mass ([MH^+]) of MET2 was estimated to be 538 using a mass spectrometry method reported previously (Sahasranaman et al., 2004). Based on the molecular mass and comparing the chromatographic elution profile of MET2 to putative metabolites (Affrime et al., 2000a), MET2 is expected to be 6β-OH-MF. This confirms other reports that have hinted the formation of this metabolite (Zbaida et al., 1997; Affrime et al., 2000b; Teng et al., 2003b; Teng et al., 2003a). Hydroxylation at the 6β-position is a common route of metabolism among glucocorticoids with budesonide, triamcinolone acetonide and flunisolide getting metabolized into their respective 6β-hydroxy derivatives (Kupfer and Partridge, 1970; Gordon and Morrison, 1978; Chaplin et al., 1980; Edsbacker et al., 1987; Jonsson et al., 1995). Using HPLC-UV detection in the present study, only the decline of MF and the formation of MET2 could be viewed. This agrees with the report by Teng et al., who monitored the formation of only 6β-OH-MF in human liver using UV and concluded that other metabolites also should be formed to

explain the loss of MF. The present study clearly reveals the existence of at least five metabolites. Thus, the use of the tritium-labeled drug provided valuable information about the formation of the other metabolites as well. MET1 is not among the putative metabolites of MF that were proposed by Affrime et al (Affrime et al., 2000a). Whether MET1, is identical to a fraction in urine reported by the same group to elute in a "very polar region" (Affrime et al., 2000a) needs further investigations. Unfortunately, we were not able to identify MET1, or MET3, MET4 and MET5, either because they could not be extracted (MET1) or were formed in insufficient quantities. In summary, these experiments showed the generation of a total of 5 MF metabolites, the identity of one (6β-OH-MF) confirmed previous suggestions (Teng et al., 2003a; Teng et al., 2003b; Davies, 2004).

In-vivo experiments in rats after iv administration of MF revealed additional information. The data obtained 2 hours post-administration showed that most of the radioactivity (~90%) is present in the stomach, intestines and the intestinal contents while radioactivity in all other organs was lower, and represented mainly MET1-3. This strongly suggests that MF and its metabolites are excreted through the bile. Not surprisingly, the majority of the radioactivity in the intestine was rather hydrophilic (MET1). Biliary excretion has also been reported for other glucocorticoids like triamcinolone acetonide and prednisolone (Kripalani et al., 1975; Mueller and Potter, 1981; Khalafallah and Jusko, 1984).

MF-related radioactivity was much lower in other tissues. The amount of radioactivity associated with the brain was negligible and the least among the organs studied. This is consistent with results from other studies that show that the uptake of glucocorticoids into the brain is limited by the multidrug resistance (mdr) P-glycoprotein (Pgp) efflux transporter at the blood-brain barrier

(Karssen et al., 2002; Arya et al., 2005). Overall these experiments clearly indicate that MF is metabolized efficiently *in vivo* and the metabolites are eliminated by biliary excretion into the intestinal tract.

The evaluation of the potential bioactivity of the formed metabolites was a significant part of this study, as Isogai reported that a number of potential MF metabolites showed distinct binding to the glucocorticoid receptor (Isogai et al., 1993). Using the selected approach of assaying HPLC fractions of liver S9 fractions for glucocorticoid binding allowed us to monitor all metabolic fractions, despite not knowing their chemical identity. Among the metabolite fractions (Table 2), two of the 5 metabolites (MET2 and to a smaller degree MET3) showed measurable affinity to the glucocorticoid receptor, while MET1 (the most hydrophilic metabolite), MET4 and MET5 did not bind. While the low affinity of MET3 and the lack of any binding of MET 1, MET4 and MET5 is in agreement with reduction of glucocorticoid bioactivity through metabolism, the RBA value of MET2 (700) is greater than the RBA values of some commonly used inhaled corticosteroids like flunisolide (RBA 180), triamcinolone acetonide (RBA 233) and is comparable to budesonide (RBA 935) (Hochhaus and Moellmann, 1990; Wuerthwein et al., 1992). The strong affinity of 6β-OH-MF is surprising because the 6β-hydroxy derivatives of other glucocorticoids do not show significant activity towards the glucocorticoid receptor. The RBA values of 6β-OH-budesonide and 6β-OH-flunisolide have been reported to be 6 and less than 1 respectively (Dahlberg et al., 1984; Hochhaus and Moellmann, 1990).

In summary**,** the original data on MF reported low systemic availability for this compound. MF produces significant systemic effects after an oral inhalation. 800 µg BID of MF administered by a metered dose inhaler for 28 days produced a 20%-30% suppression of the hypothalamic-

pituitary-adrenal axis (HPA) compared to placebo (P<0.05) (Affrime et al., 2000b). By comparison, a 880 µg BID of fluticasone propionate also delivered via pMDI produced a 43%-56% suppression of HPA compared with placebo (P<0.01) (Affrime et al., 2000b; Crim et al., 2001). In a recent study, fluticasone propionate and MF delivered via their respective DPIs showed similar dose dependent cortisol suppressions (Fardon et al., 2004)). The formation of the active metabolite, 6β-OH-MF, might explain in part the systemic effects after MF administration if these metabolites. Recently, other studies presented in abstract form have reported a systemic availability of 5% (Derks, 2005). Systemic effects of MF might therefore be the result of both a higher systemic exposure than originally assumed and the presence of active metabolites. Therefore, the measurement of this metabolite is critical in obtaining a true measure of the systemic bioavailability of mometasone furoate.

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Footnote

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Legends for figures

Scheme 1. A schematic degradation pathway for conversion of MF into its degradation products D1, D2 and D3 on incubation of MF (C_0 = 6.2 µM) in plasma at 37°C for 72 h. Structures A, B, D and E represent MF, D1, D2 and D3 respectively. Structure C shows only D-ring of the steroid for presenting the proposed reaction mechanism for the conversion of B to D. A similar reaction mechanism would be possible for formation of structure E from A. Broken arrows indicate the possible formation of D2 from D3.

Figure 1. Concentration-time profile of MF and its degradation products following incubation of MF (C_0 = 6.2 µM) at 37°C in (a) rat plasma and (b) human plasma for 72 h (n=3).

Figure 2. Representative radiochemical elution profiles of [1,2-^{3}H]-MF incubated with S9 fraction of rat lung at 37°C (A) at time zero, (B) after 2 h at 37°C and (C) after 24 h

Figure 3. Representative radiochemical elution profiles of (a) [1,2-^{3}H]-MF incubated in S9 fraction rat liver at time zero and (b) [1,2-^{3}H]-MF incubated in S9 fraction of rat liver for time 20 min at 37°C (bars in black and gray represent MF in S9 fraction of rat liver and homogenizing buffer respectively. See formation of various metabolites (MET1-5)).

Figure 4. Concentration-time profile of [1,2-^{3}H]-MF following incubation of [1,2-^{3}H]-MF in S9 fraction of rat liver and (b) concentration-time profile of the metabolites formed after incubation of ^{3}H-MF in S9 fraction of rat liver at 37°C for 1 h.

Figure 5. Concentration-time profile of [1,2-^{3}H]-MF following incubation of [1,2-^{3}H]-MF in S9 fraction of human liver and (b) concentration-time profile of the metabolites formed on incubation of MF in S9 fraction of human liver at 37°C for 1 hr.

Figure 6. Radiochemical elution profiles of intestinal contents two hours after intravenous administration of 5 µCi [1,2-^{3}H]-MF in male Sprague-Dawley rats.

Figure 7. Competitive binding experiments to the glucocorticoid receptor in rat lung cytosol (n=2). Nonlinear regression of non-transformed data was used for the determination of IC_{50} values of dexamethasone, MF, MF_{frac}, metabolites MET2 and MET3

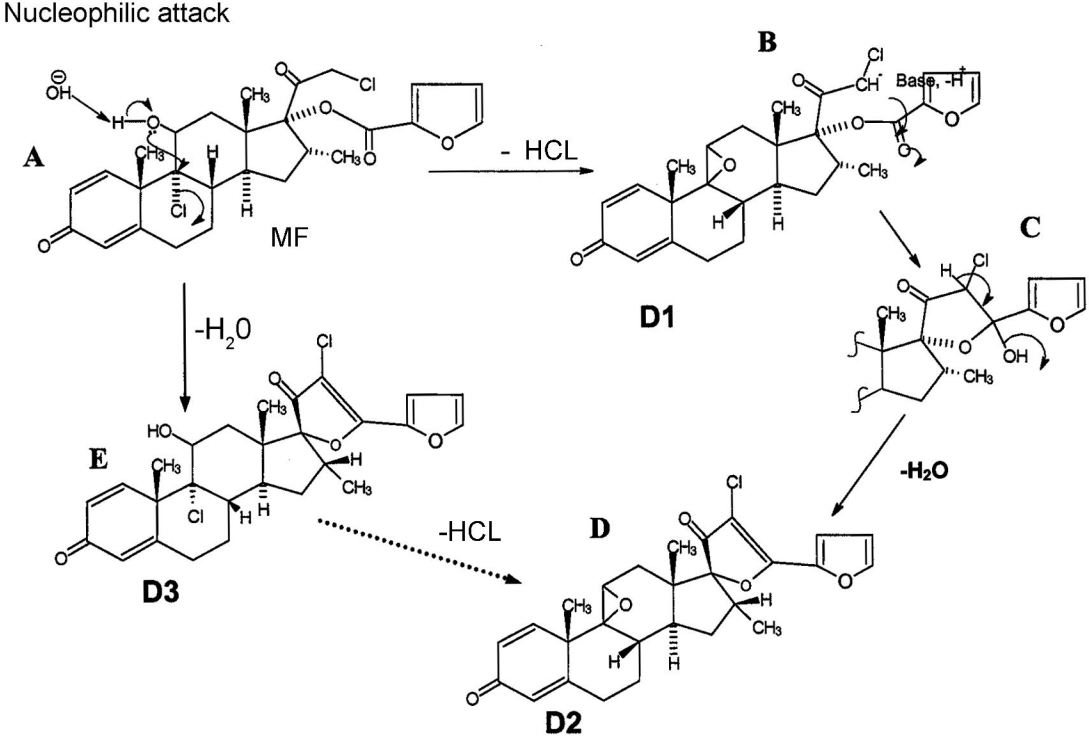
Table 1. Percentage of dose distributed into different tissues two hours after intravenous administration of [1,2-^{3}H]MF in male Sprague-Dawley rats.

Tissue	**% of dose (n=3)**
Brain	0.05±0.01
Thymus	0.09±0.03
Heart	0.07±0.02
Spleen	0.27±0.11
Lung	0.35±0.03
Kidney	0.28±0.04
Stomach	1.22±0.32
Liver	1.99±1.18
Large intestine	2.45±1.77
Small intestine	4.51±0.10
Intestinal contents	81.47±5.69
Muscle	0.21±0.06[a]
Fat	0.41±0.18[a]
Skin	0.05±0.02[b]
Plasma	0.25±0.07[c]
Urine	0.08±0.02[c]

[a] % of dose/gram tissue
[b] % of dose/cm^2
[c] % of dose/ml

Table 2. Average Relative binding affinities (RBA) to the glucocorticoid receptor of rat lung tissue.

Compound	**RBA**
Dexamethasone	100
Mometasone furoate	2900
MF_{frac}	2700
MET1	<25
MET2	700
MET3	25
MET4	<50
MET5	<50



Scheme I

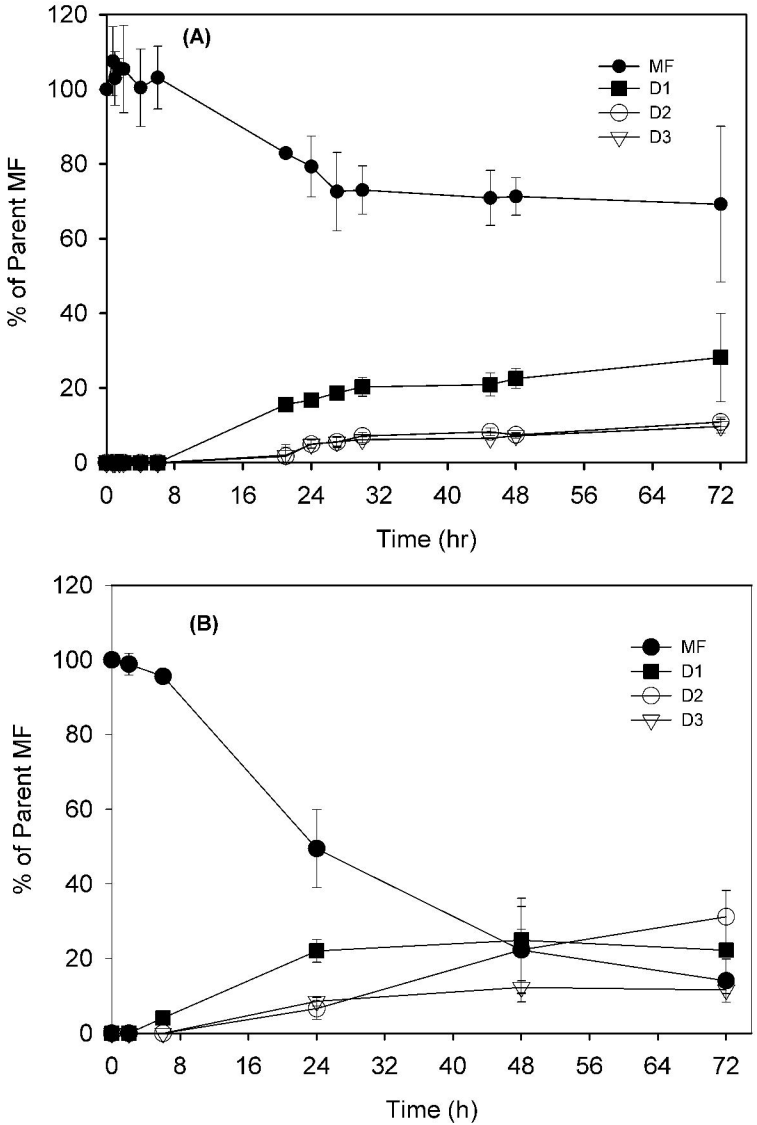
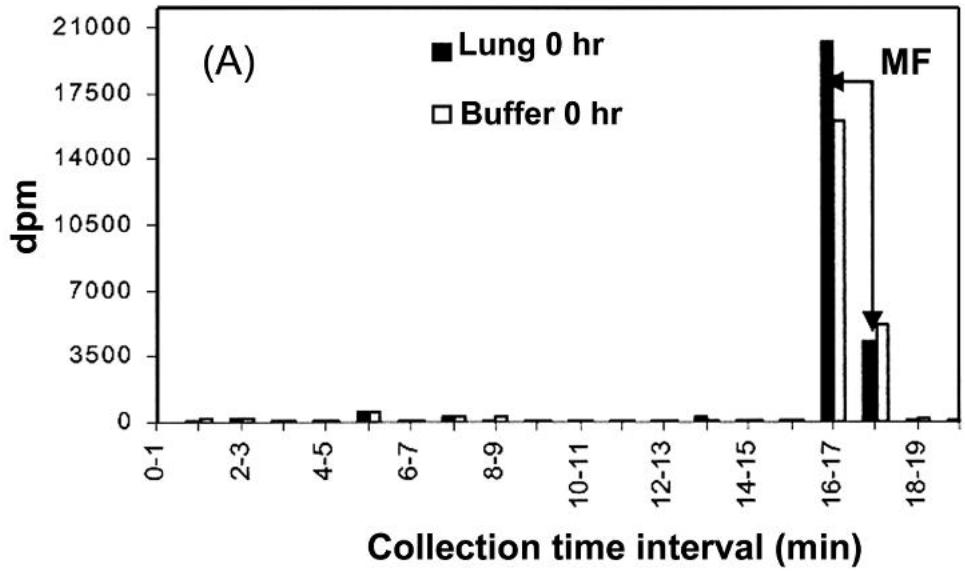
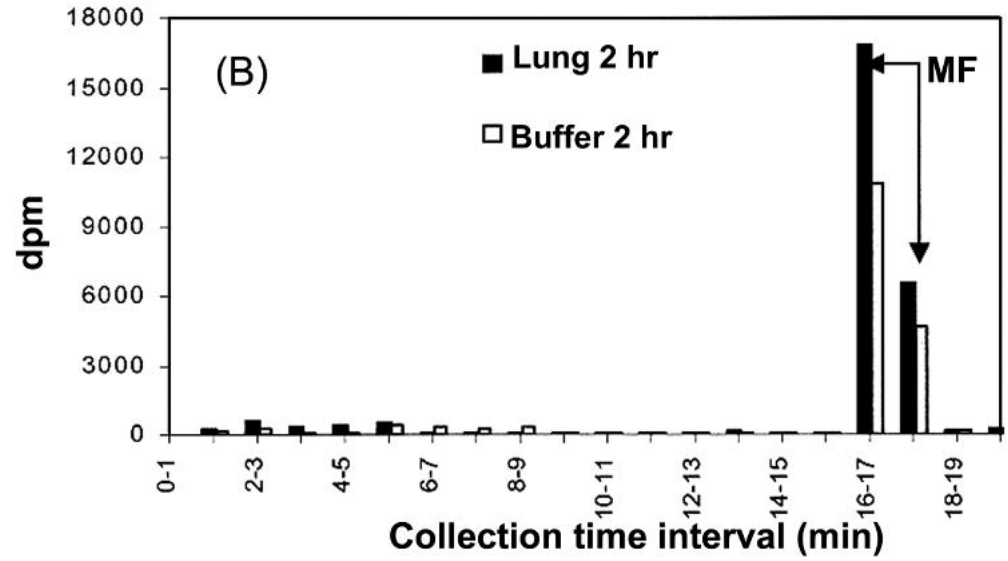


Figure 1


(A)
Lung 0 hr
Buffer 0 hr
MF
dpm
21000
17500
14000
10500
7000
3500
0
0-1
2-3
4-5
6-7
8-9
10-11
12-13
14-15
16-17
18-19
Collection time interval (min)


(B)
Lung 2 hr
Buffer 2 hr
MF
dpm
18000
15000
12000
9000
6000
3000
0
0-1
2-3
4-5
6-7
8-9
10-11
12-13
14-15
16-17
18-19
Collection time interval (min)

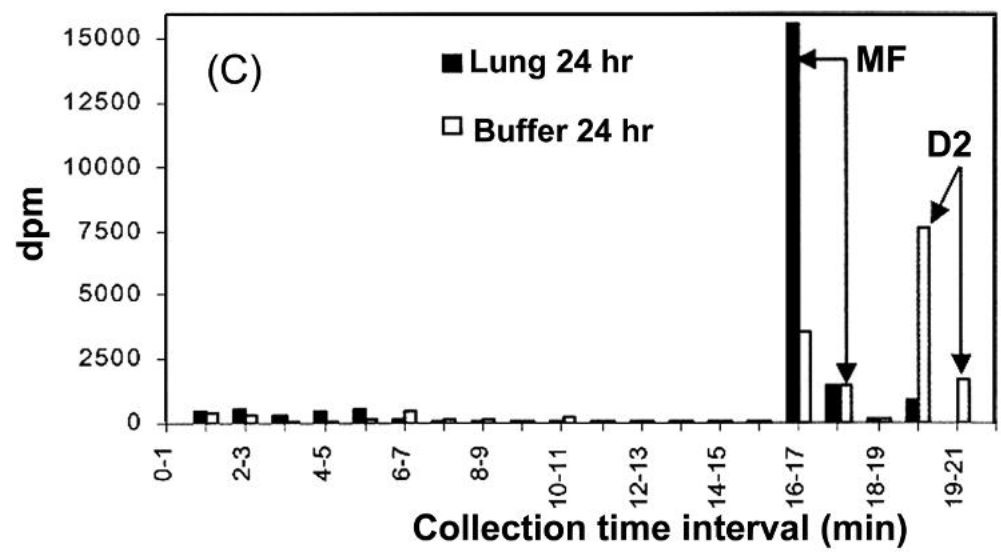
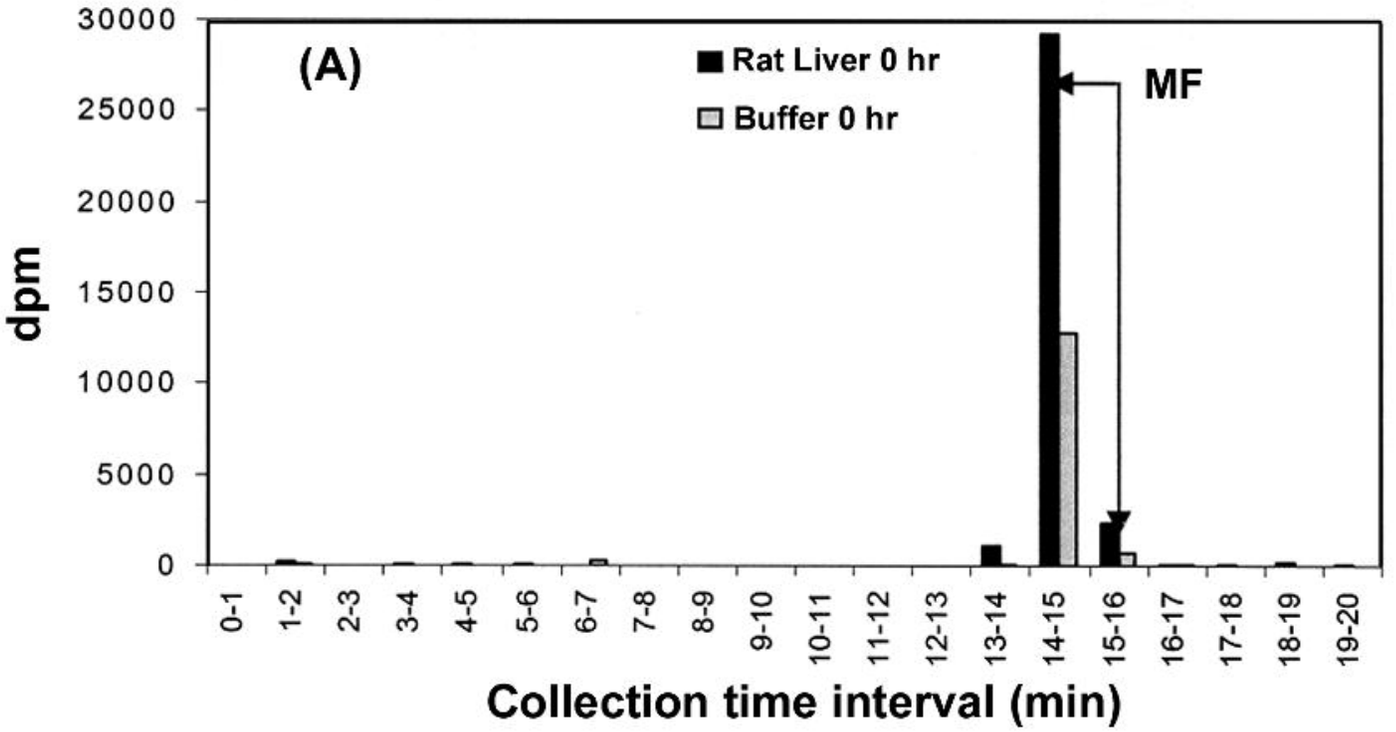

(C)
Lung 24 hr
Buffer 24 hr
MF
D2
dpm
15000
12500
10000
7500
5000
2500
0
0-1
2-3
4-5
6-7
8-9
10-11
12-13
14-15
16-17
18-19
19-21
Collection time interval (min)

Figure 2


(A)
Rat Liver 0 hr
Buffer 0 hr
MF
dpm
30000
25000
20000
15000
10000
5000
0
0-1
1-2
2-3
3-4
4-5
5-6
6-7
7-8
8-9
9-10
10-11
11-12
12-13
13-14
14-15
15-16
16-17
17-18
18-19
19-20
Collection time interval (min)

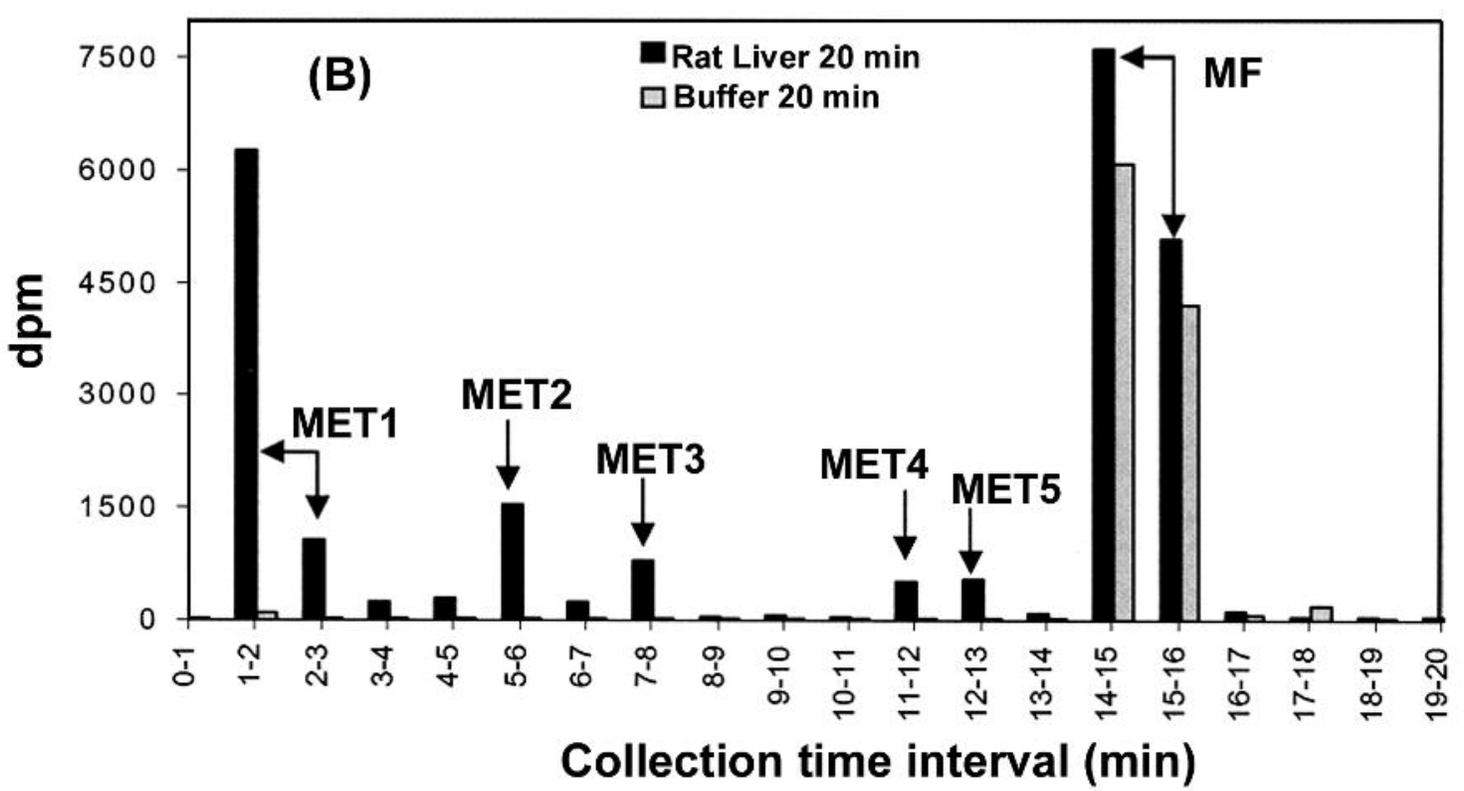

(B)
Rat Liver 20 min
Buffer 20 min
MF
MET1
MET2
MET3
MET4
MET5
dpm
7500
6000
4500
3000
1500
0
0-1
1-2
2-3
3-4
4-5
5-6
6-7
7-8
8-9
9-10
10-11
11-12
12-13
13-14
14-15
15-16
16-17
17-18
18-19
19-20
Collection time interval (min)

Figure 3

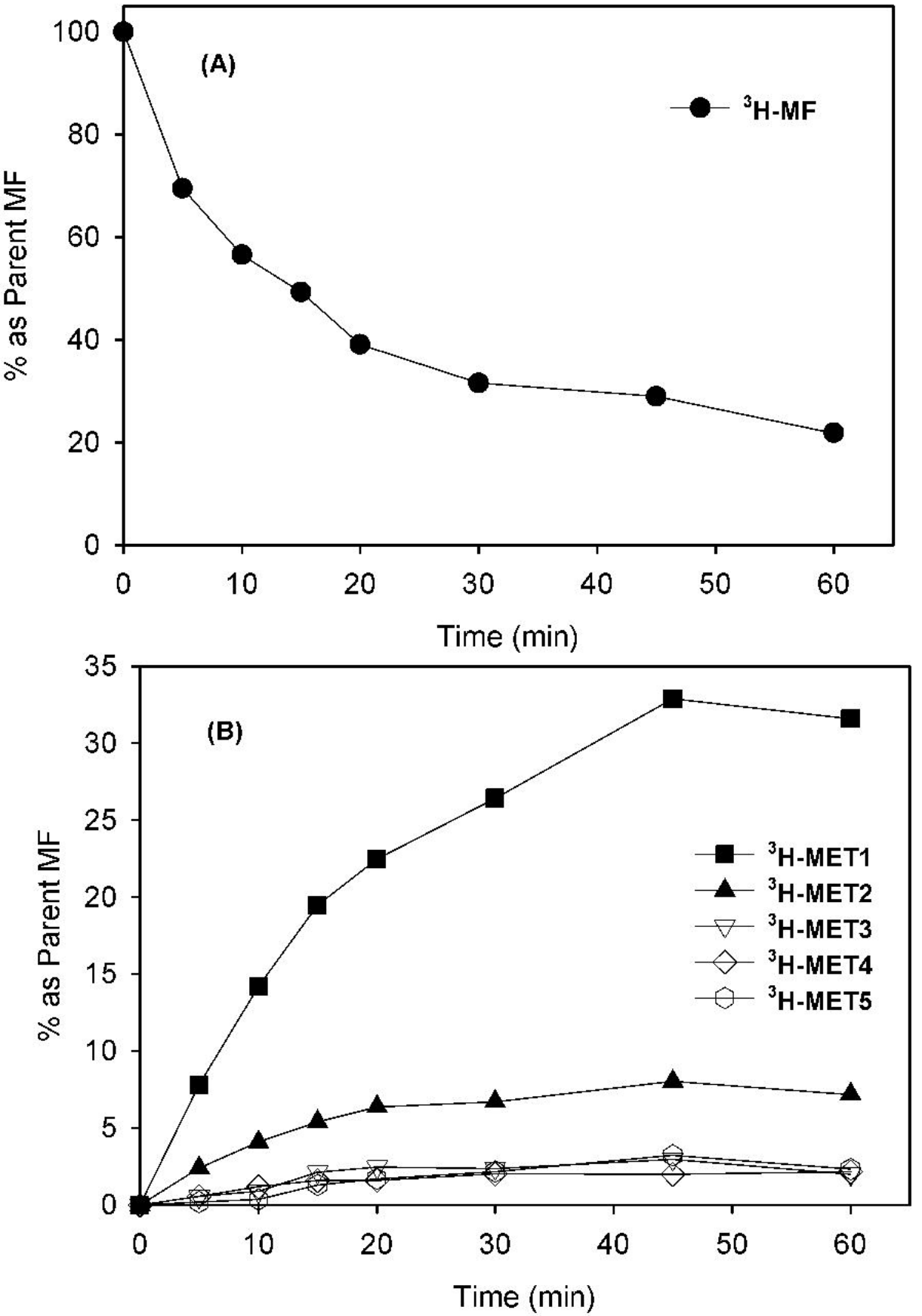
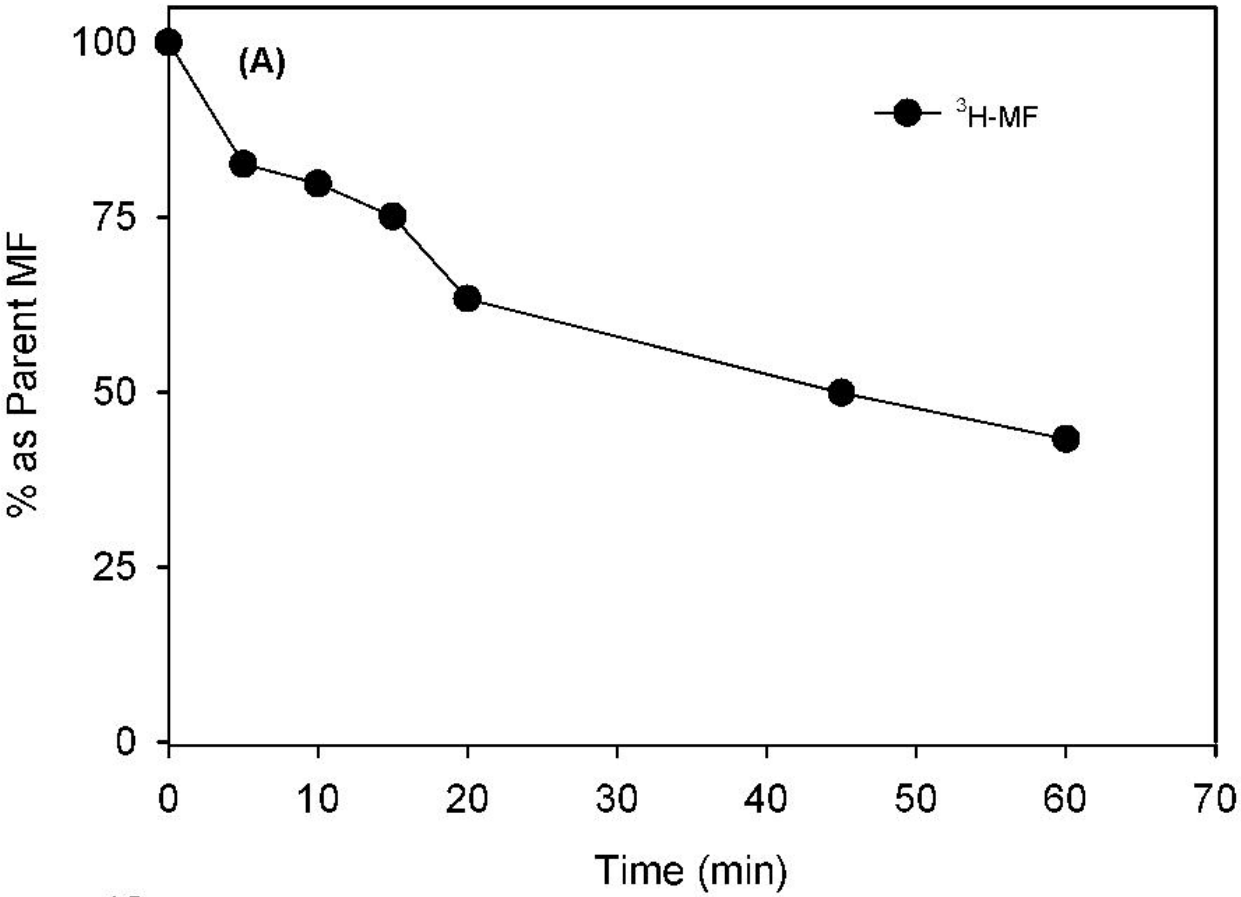

(A)
^{3}H-MF
% as Parent MF
Time (min)
(B)
^{3}H-MET1
^{3}H-MET2
^{3}H-MET3
^{3}H-MET4
^{3}H-MET5
% as Parent MF
Time (min)

Figure 4


(A)
^{3}H-MF
% as Parent MF
100
75
50
25
0
0
10
20
30
40
50
60
70
Time (min)

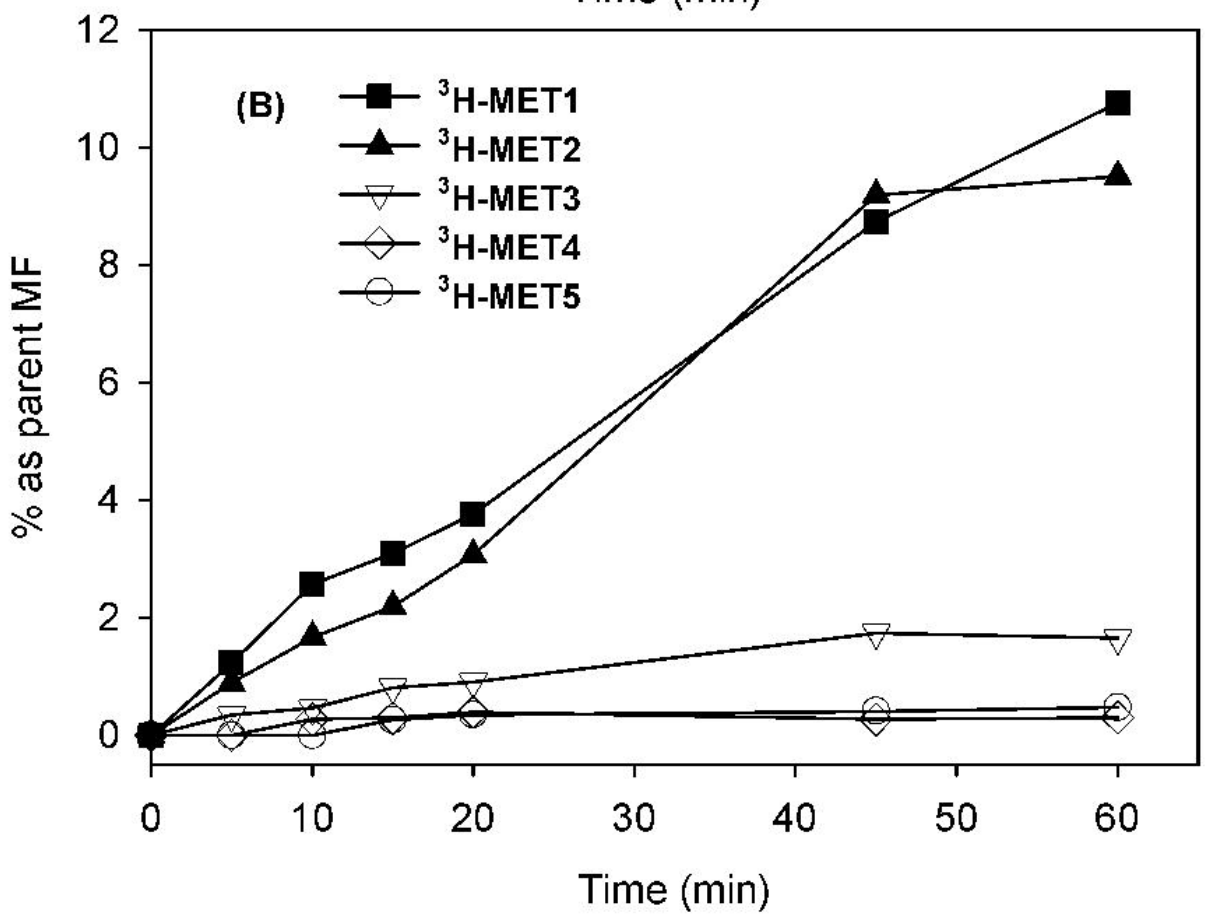

(B)
^{3}H-MET1
^{3}H-MET2
^{3}H-MET3
^{3}H-MET4
^{3}H-MET5
% as parent MF
12
10
8
6
4
2
0
0
10
20
30
40
50
60
Time (min)

Figure 5

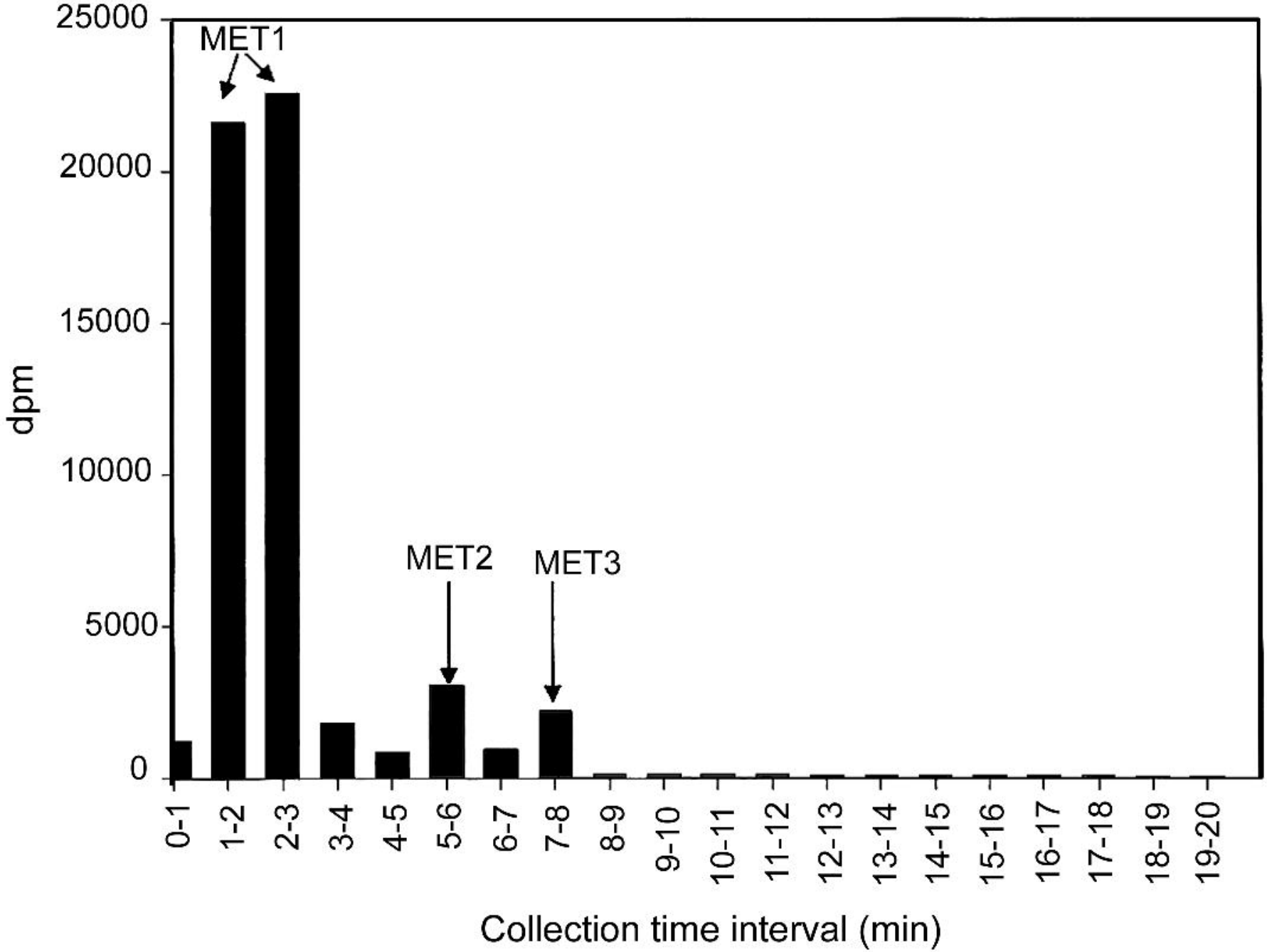

25000
20000
15000
10000
5000
0
dpm
MET1
MET2
MET3
0-1
1-2
2-3
3-4
4-5
5-6
6-7
7-8
8-9
9-10
10-11
11-12
12-13
13-14
14-15
15-16
16-17
17-18
18-19
19-20
Collection time interval (min)

Figure 6

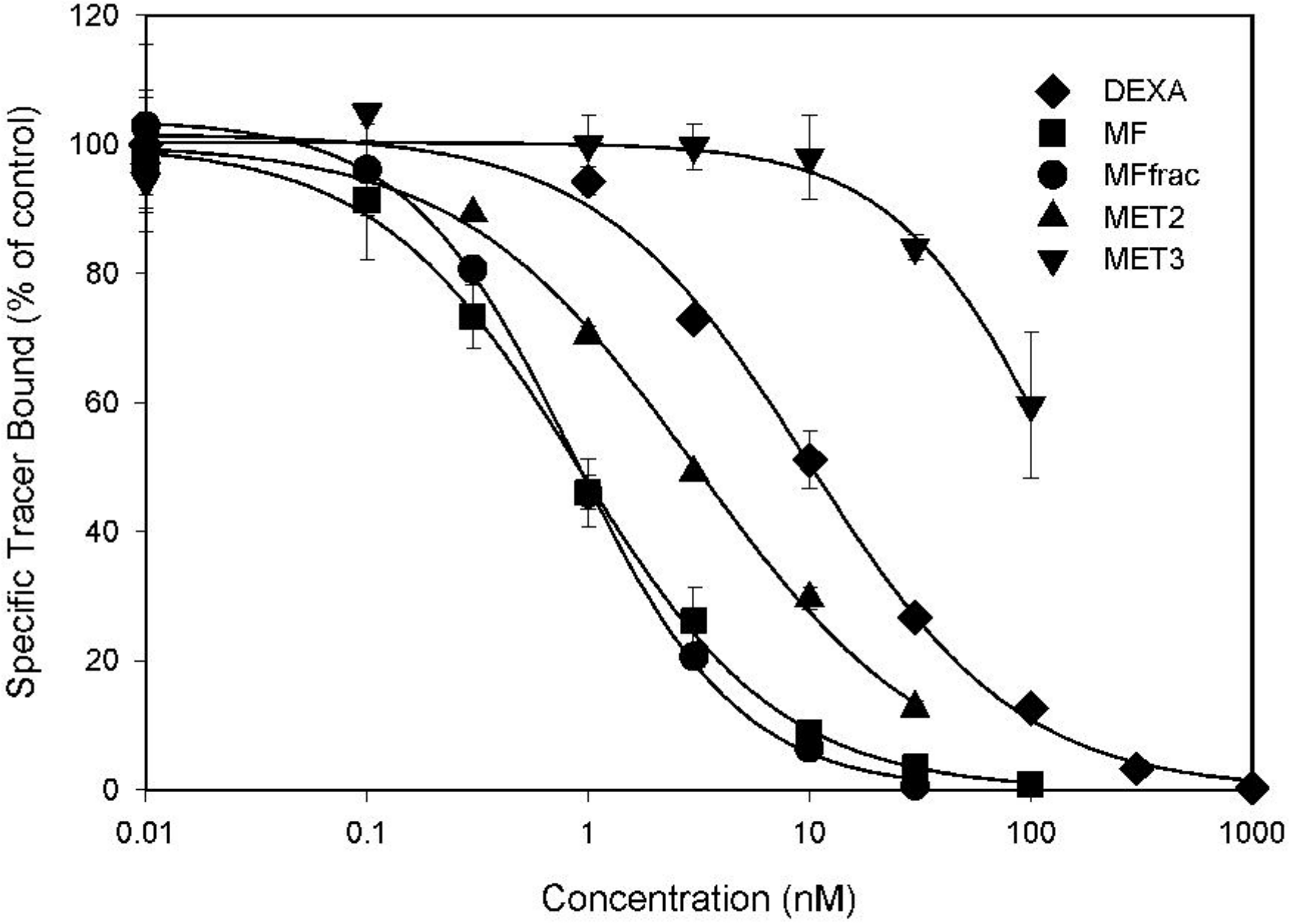

120
100
80
60
40
20
0
Specific Tracer Bound (% of control)
0.01
0.1
1
10
100
1000
Concentration (nM)
DEXA
MF
MFfrac
MET2
MET3

Figure 7