る．実際のTIMESソフトウェアでは代謝物の予測だけでなく，彼らによって同じく開発されたQSARモデルであるOASIS（Optimized Approach Based on Structural Indices Set）との組合せによって，個々の代謝物のエームス試験，染色体異常試験結果まで予測することができる．

おわりに

(Q)SARモデルの*in vitro*遺伝毒性試験の予測に関して本稿で述べた．しかしながら，*in vitro*遺伝毒性試験自体が発がん性化学物質の予測を目的とするスクリーニング試験であることを考えると，スクリーニング試験の結果の予測を行うことにどれだけの科学的な価値があるか，疑問に思われるかも知れない．医薬品を始め，多くの化学物質の安全性評価のためにエームス試験，染色体異常試験が法律で義務づけられており，多数の候補化合物や，既存化学物質の中から優先度の高い物質を選択するツールとして(Q)SARは現実的に極めて有効である．しかしながら，(Q)SAR研究の本質はツールとしての利用ではない．始めに述べたように(Q)SARはMillerの唱えた発がん化学物質の求電子理論により，エームス試験というバイオアッセイの結果を証明することから始まった．この思想は分子レベルで生命現象，特に毒性メカニズム解明を試みた最初の例である．ほとんど全ての毒性はエームス試験のように単純では無く，極めて複雑であり，またメカニズムが不明である．2007年から米国EPAが中心となって開始したToxCastプログラムでは*in vitro*試験系から多くの毒性経路やメカニズムを抽出し，(Q)SARに組み入れる手法を開発する[11]．成功すれば，Millerのように様々の毒性を分子レベルで解明できるかも知れない．それが(Q)SARとコンピュータトキシコロジーの最終目標であり，その利用が科学的知見に基づく化学物質の真のリスク評価に通じるものであると信じる．我が国でも同様のプロジェクトを立ち上げ，毒性学の未来を切り開く努力が必要である．

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4）*in vivo*反復投与毒性の構造活性相関による予測評価の展望

小野 敦

Perspective of predictive toxicity assessment of *in vivo* repeated dose toxicity using structural activity relationship

Atsushi Ono

Tens of thousands of existing chemicals have been widely used for manufacture, agriculture, household and other purposes in worldwide. Only approximately 10% of chemicals have been assessed for human health hazard. The health hazard assessment of residual large number of chemicals for which little or no information of their toxicity is available is urgently needed for public health. However, the conduct of traditional toxicity tests which involves using animals for all of these chemicals would be economically impractical and ethically unacceptable. (Quantitative) Structure-Activity Relationships [(Q)SARs] are expected as method to have the potential to estimate hazards of chemicals from their structure, while reducing time, cost and animal testing currently needed. Therefore, our studies have been focused on evaluation of available (Q)SAR systems for estimating *in vivo* repeated toxicity on the liver. The results from our preliminary analysis showed the distribution for LogP of the chemicals which have potential to induce liver toxicity was bell-shape and indicating the possibility to estimate liver toxicity of chemicals from their physicochemical property. We have developed (Q)SAR models to *in vivo* liver toxicity using three commercially available systems (DEREK, ADMEWorks and MultiCASE) as well as combinatorial use of publically available chemoinformatic tools (CDK, MOSS and WEKA). Distinct data-sets of the 28-day repeated dose toxicity test of new and existing chemicals evaluated in Japan were used for model development and performance test. The results that concordances of commercial systems and public tools were almost same which below 70% may suggest currently attainable knowledge of *in silico* estimation of complex biological process, though it possible to obtain complementary and enhanced performance by combining predictions from different programs. In future, the combinatorial application of *in silico* and *in vitro* tests might provide more accurate information which support regulatory decisions. At the same time, an appropriate strategy to use (Q)SAR for of the efficiency and accuracy in chemical management is necessary.

Keywords: Quantitative Structure-Activity Relationships, repeated dose toxicity, liver, existing chemicals, risk assessment

1．はじめに

現在，日本国内では約10万種もの化学物質が様々な用途で流通していると言われている．そのうち，医薬品や農薬など用途ごとに規制されている物質以外の一般化学物質については，主に「化学物質の審査及び製造等の規制に関する法律」（以下「化審法」）により規制される．化審法では，新規の化学物質が製造・輸入される際には，物性（分解性・蓄積性），人への毒性及び生態毒性について，それぞれ定められた試験による審査が行われており，人への毒性に関しては「細菌を用いる復帰突然変異試験」，「ほ乳類培養細胞を用いる染色体異常試験またはマウスリンフォーマTK試験」及び「28日間反復毒性試験」の３種のスクリーニング毒性試験による安全性

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評価が実施されている．一方，昭和48年の化審法制定以前から流通していた化学物質（既存化学物質）については，国で必要な試験を実施して安全性評価を進めているが，我が国だけでも数万種の既存化学物質が流通しており，これまでに安全性評価が終了した化学物質はごく一部に過ぎず，残された既存化学物質は人への安全性について不明のまま流通している．それらの化学物質が直ちに問題を起こすことはないとしても潜在的なリスクとなっており，国民の健康を守るためには早急な安全性の評価が望まれるが，数万種の化学物質について従来実施されている毒性試験を実施するのは必要となる費用や期間の面から現実的ではなく，特に動物試験については動物愛護の観点からもなるべく最小限に留める事が求められており，効率的で信頼性の高い新たな安全性評価手法の確立が望まれている．近年，既に毒性試験が実施されている構造類似物質の毒性情報から評価物質の毒性を類推するカテゴリーアプローチ，さらには，化学物質構造と毒性発現との相関から化学物質の毒性を予測する（定量的）構造活性相関（(Quantitive) Stracture Activity Relationship）手法が解決策の一つとして期待されており，世界各国で検討が進められている[1]．化学物質の構造から毒性を予測することが出来れば，毒性試験未実施の化学物質についても安全管理が可能となり，毒性試験を必要とする化学物質の優先順位付けや絞り込みを行うことで効率的な安全性評価が可能となる．さらに将来的には，予測精度の向上により試験そのものが必要なくなる日も来るかもしれない．

2．構造が類似する化学物質からの毒性予測

(Q)SARの基礎は「類似の構造をもつ化学物質は類似の生理活性を生じる」という概念である．医薬品開発のエキスパートは，化学構造から"既知の"薬効や物性をある程度推測出来るという話もあるが，毒性に関してはどうであろうか？ Fig. 1に示す９種のクロロニトロベンゼンの同族体は，いずれも既存化学物質である．これらのうち物質４（2,4-ジクロロニトロベンゼン（CAS No. 611-06-3））については，既存点検による「ラットを用いた反復経口投与毒性・生殖発生毒性併合試験」が実施されており，その結果，血液，腎臓，肝臓への障害が認められ，NOEL 8mg/kg/day未満と報告されている．また，物質２（1,4-ジクロロ-2-ニトロベンゼン（CAS No. 89-61-2））については，既存点検による「ラットを用いる経口投与簡易生殖毒性試験」結果から雌の生殖に関するNOEL 20 mg/kg/dayと報告がある．物質１と３については，毒性試験は実施されてはいないが，物質１～４は化審法では区別されておらず，ジクロロニトロベンゼンとしていずれも第２種監視物質に指定されている．一

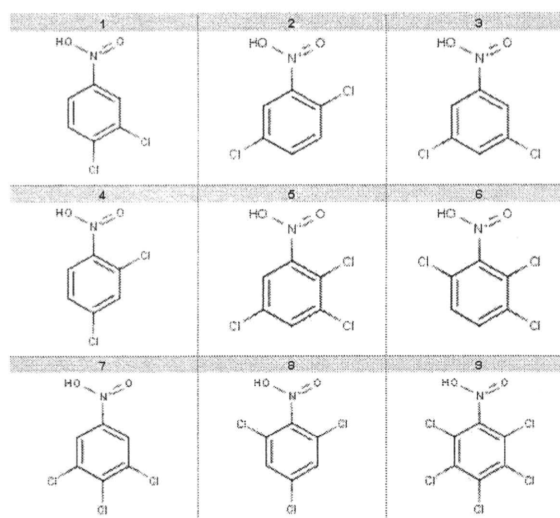

Fig. 1 Structurally similar nine chloronitrobenzene homologs

方，物質９（ペンタクロロニトロベンゼン（CAS No. 82-68-8））は，過去に土壌殺菌剤，防かび剤，防汚剤に使用されていた農薬で，ビーグル犬を用いた混餌投与による２年間慢性毒性試験により胆汁うっ滞性肝障害などから NOEL 0.75 mg/kg/dayと報告されている．この物質は，農薬としては販売禁止となっており厳しい残留基準が定められている．では，物質５～８はどうであろう．これらの物質について毒性試験は実施されていない．しかし，物質構造から物質１～４及び９と同程度の毒性が懸念されないだろうか．もちろん，予想外に毒性が低い可能性も否定出来ない．ここで問題なのは，これらの物質のように安全性未評価の既存化学物質は毒性試験が実施されない限り，特に規制も受けずに流通可能なことである．環境中に一度大量に排出されてしまった化学物質を除去するのは非常に困難であるが，安全性未評価の化合物について一律に厳しい規制を実施するのは現実的ではない．カテゴリーアプローチや（Q)SAR手法による安全性評価スキームが実用化されれば，毒性試験が実施されていない化学物質についても適切な管理を行うことが可能である．Fig. 1の物質群については，化学物質の毒性評価についてある程度の経験があれば，その毒性を疑うであろう．しかし，経験に基づく評価は，評価者によって判断が違ってしまう可能性があるため安全性評価に用いるためには，評価基準が明示的に示されている必要がある．化学構造と毒性との関係については，1970年代から研究されている．1972年にCramerらは，82化合物の構造とNOELデータの解析結果から化学物質の毒性を３クラスに分類する33の構造ルールからなるYes/No型の決定樹を発表している[2]．さらに，1996年Munroらは600以上の化学物質のNOELによるCramer

ruleの検証を行い新たに5つのルールを追加した[3]．JECFAでは毒性情報が無い既存食品香料の安全性評価におけるMunroらの修正Cramer ruleの適用を検討している[4]．Cramer ruleでは，Fig. 1の物質は全てクラス3（強い毒性が懸念される物質）と判定される．

3．化学物質の物理化学的性質と毒性

(Q)SARでは，化学物質の構造そのものだけでなく化学構造から計算可能な様々な物理化学的性質や化学構造の情報を数値化して記述子として予測に用いる．化学物質が生理活性を生じるためには，生体に吸収され，作用部位に到達する必要がある．化学物質の腸管吸収性や細胞膜透過性は，医薬品開発においてバイオアベイラビリティーの向上に重要であり，数多くの研究から化学物質の脂溶性すなわち水／オクタノール分配係数（LogP）との関係が示されている[5,6]．逆に一般化学物質では，吸収率が高ければ経口投与による毒性が発現する可能性が高い．Fig. 2に化審法で実施された28日間反復投与動物試験により肝毒性が認められた物質のLogPの区間分布を示す．上側パネルは，新規化学物質として申請された物質，下側パネルは既存化学物質点検により試験が実施された物質であり，いずれの物質群においても明らかにLogP= 3付近を中心に肝毒性を有する物質の割合が多いことがわかる．しかも，その分布は2つのデータセットでほぼ共通していることから化学物質構造によらない共通ルールであり，化学物質の物理化学的性質からの毒性予測の可能性を示唆している．本来，LogPは実験

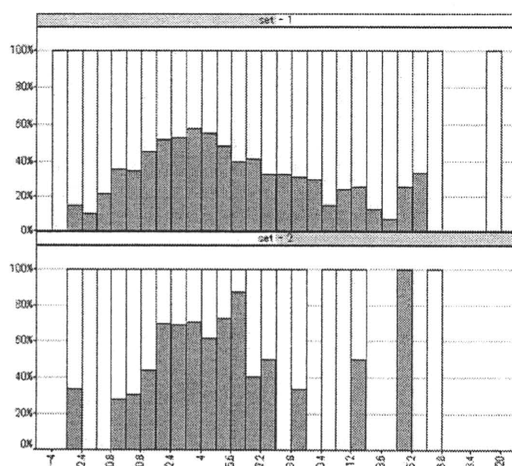

Fig. 2 LogP distribution of liver toxic chemicals X axis showing calculated LogP values (XLogP) and Y axis showing relative percentage of chemicals with (gray) and without (white) the liver toxicity in 28-day repeated dose toxicity test using Rat.: Upper panel (Set-1): Data of newly registered 1227 chemicals for Japanese Chemical Substances Control Law, Lower panel (Set-2): Data of Japanese existing 251 chemicals tested by Ministry of Health, Labour and Welfare.

的に求められる物性値であるが，化学物質の生体内での様々な挙動と関連することから様々な予測計算法が報告されており，今日では，かなりの精度で予測計算が可能である．すなわち，LogP計算こそが構造活性相関の最大の成功例ともいえる．今日では，LogPは多くの構造活性相関モデルでパラメータとして用いられており，経済産業省で検討されている蓄積性予測モデル[7]や環境省で検討されている生態影響予測モデル[8]は，いずれも化学物質を幾つかの構造群に分類し，構造群ごとにLogPとの回帰式をもとに予測を行うものである．しかし，計算により求められるLogPはあくまで予測値であり，化学構造によってアルゴリズム間で異なる値となることに注意が必要である[9]．なお，図2の解析にはLogP予測計算値としてXLogPを用いている[10]．

4．代表的な構造活性相関手法による*in vivo*毒性予測精度の検証

上述の例は，*in vivo*反復毒性についても化学構造や構造から計算可能な性質から予測出来る可能性を示しているが，これまでのところ多岐に渡る化学物質に適用可能な*in vivo*反復毒性について精度の良い（Q)SARモデルは報告されていない．我々の研究室では，これまで化審法における新規化学物質審査や既存化学物質点検における安全性評価を実施するとともに試験データのデータベース化を進めてきており，蓄積されたデータ（化審法データ）をもとに，平成15年度より人健康影響に関する3種のスクリーニング毒性試験についての（Q)SAR評価法の開発を変異遺伝部等との共同により進めており，これまでにAmes試験については国際的評価が高い3種のアプローチの異なるソフトウェアを組み合わせることで実用可能なレベルの予測が可能であることが示された[11]．現在，引き続き染色体異常試験及び28日反復投与毒性試験について検討を進めており，以下にAmes試験予測に用いた3種のソフトウェアを用いて28日反復投与毒性予測の検討を行った結果をそれぞれの手法の特徴とともに示す．

4．1　DEREKによる肝毒性アラートの構築

毒性予測の分野における（Q)SARは，Cramer ruleのように専門家による知見をもとに判断を行うエキスパートシステムと化合構造もしくはその部分構造から計算される物性値などの特徴量を記述子として統計的・情報数理的に解析する手法に分類される．DEREKは，文献情報や有識者により経験的に得られている毒性部位の部分構造（アラート）と経験則をルール化した知識ベースにより，定性的な毒性予測を行うエキスパートシステムである[12]．エキスパートシステムでは，高度な定量予測を

行うのは難しいが，予測の根拠（知識ベース）が容易に確認出来ることやモデル全体の構造を変えることなく知識ベースの追加拡張を行うことが出来る等の利点がある．DEREKを開発したLhasa社は，様々な機関や企業等との共同研究により構築された有害性アラート（部分構造）を知識ベース化してDEREKのアラートとして提供しており，我々はLhasa社と共同で化審法データを基に新規肝毒性アラートの構築を行っている．これまでの研究では，約800化合物の構造情報と毒性試験成績をもとに，(i)化合物構造クラスタリング，(ii)視覚的解析，(iii)関連する毒性に対してのDEREKの既存アラートとの比較により，新たに34個の新規肝毒性Rapid Prototypeアラートの構築に成功している．共同研究を始めた当初，DEREKには40種の肝毒性アラートが搭載されていたが，新規アラートの追加により，アラート構築に用いた学習セット（化審法データ）に対する予測精度は，感度（陽性物質のうち陽性判定された割合）が23%から61%に上昇し，全体の一致率（的中率）も61%から80%に上昇した．しかし，独立した外部データセットによる検証の結果は，感度31%，一致率58%であった．DEREKやCramer ruleのようなエキスパートシステムでは，有害性アラートとの部分構造マッチングにより判定を行うため，有害性アラートがカバーしていない毒性物質は偽陰性判定されてしまう．また，新たに抽出した肝毒性Rapid Prototypeアラートについて解析したところ，幾つかのアラートは非常に少ない物質に由来しており，それらの一部が検証データにおいて非常に多くの物質で偽陽性判定をしていることが一致率の低さの一因であると判断された．現在，構造アラートとのマッチングと分子量やLogPを組み合わせた予測精度向上の検討を進めている．

4．2 ADMEWorksによる肝毒性判別モデルの構築

ADMEWorksは，2次元的分子構造及びクラス特異的部分構造から計算された記述子を使って構築された判別式をもとに毒性評価を行うシステムで，構築済みモジュールにより予測を行うAdmeworksと予測モデル構築を行うモデルビルダーから構成される．モデルビルダーには400種類以上の化合物パラメータ計算モジュールが組み込まれており，部分構造パラメータを含めると数千種のパラメータを発生させることが可能である．P.C.JursらのグループがA開発したADAPT（Automated Data Analysis and Pattern recognition Toolkit）によりSupport Vector Machine（SVM）やAdaBoost（ADA）等の線形判別関数及び線形重回帰モデルを用いた予測を行う[13]．化審法で実施された28日間試験報告書等からモデル構築用にデータ整理が終了した794物質から構造の重複や不正な168物質を除外した626物質をランダムに学習セット：検証セット＝8：2に分割し，モデルビルダーを用いて肝毒性予測モデルを構築し検証を行った．それぞれについて量子力学的パラメータを含む401化学的パラメータと124部分構造パラメータ及び，1000以上の数値変換関数パラメータを利用して，計1631パラメータを発生させ，ADA等によりモデル作成に最適なパラメータの組み合わせを検証して，肝毒性予測に有効な40パラメータを選定した後，SVM，KNN（K Nearest Neighbor）及びADAの3つの判別手法によりモデル構築を行った．学習セット及び検証セットについてのそれぞれの予測手法による予測成績をTable. 1に示した．学習セットではいずれの手法でも80%以上の予測精度が得られたが，検証セットに対する予測では，ADAモデルが一致率70.3%で最も精度が高く，SVM，KNNモデルは，ADAモデルに比べて感度が低い結果であった．

4．3 MultiCASEによる肝毒性予測モデルの構築

Multiple Computer Automated Structure Evaluation（MultiCASE）は，Klopmanらよって開発された独自のCASE理論に基づくシステム[14]であり，入力分子を2～10原子（水素原子を含まない）のフラグメントに分解し，統計処理により有意に活性と相関している部分構造（BIOPHORE）を検出し，同じBIOPHOREを持つ化合物セット内における活性の違いを記述子（MODULATOR）により数値化して活性予測を行う．新規化学物質1231個のデータを学習セットとして28日間試験におけ

Table 1 Comparison of different discriminative methods on the predictive performance of liver toxicity SAR models using ADMEworks

	Training set			Test set		
No. of chemicals*	244/254			53/75		
Discriminator type	SVM	KNN	ADA	SVM	KNN	ADA
Concordance	99.2	83.9	82.9	66.4	62.5	70.3
Sensitivity	100.0	85.7	80.7	24.5	34.0	69.8
Specificity	98.4	82.3	85.0	96.0	82.7	70.7

*Positive for liver toxicity/Negative for liver toxicity

る肝毒性LOAEL値をターゲットとしてモデル構築を行い，既存化学物質251個のデータを検証セットとして予測を行った結果，感度8.7%，特異度89.2%と，ほとんどの化合物が陰性判定される結果となった．MultiCASEでは，ある程度のトレーニングデータが与えられた場合，偽陽性は少ないと考えられるが，学習セット中に評価化合物中のactiveなBIOPHOREが含まれていない場合には，偽陰性となりやすい．そこで，新規化学物質1231個のみを分子量等を基準として学習セット，検証セットに分割し，さらに陰性陽性の判定基準数パターンについてモデル構築を試みた．しかし，あまり一致率の高い結果を得ることは出来ず，一致率は最大で62%，感度は70%に達しなかった．ADMEWorks やMultiCASEのように数学・統計学的手法を用いる手法では定量的なモデル構築が可能で，かつ人為的なバイアスは排除されるが，毒性専門家の経験的な知見を反映することは難しい．また，膨大な数の記述子を機械的に生成して選別を行うため学習の過程で偶然の相関によるモデルが構築される可能性がある．

5．オープンアクセスな構造活性相関ツールの必要性

上記検討に用いたソフトウェアは，いずれも市販のものである．市販ソフトウェアは，優れた機能を有しているが，その一方でモデル構築に用いたソフトウェアを用いずに同じモデルや予測結果を得ることはほとんど不可能であり，さらに継続的な利用のためには，毎年高額なライセンス料が必要となる．今日ではコンピュータの進歩とケモインフォマティックス研究の進展により，様々な化学計算ソフトウェアがオープンソースで開発もしくは無料公開されており，それらを組み合わせて（Q)SARモデルを構築することも可能である．そこで，パブリックで利用可能なツール用いて行った検討結果を以下に示す．MultiCASEで用いたデータセットについて，構造記述子による予測モデルと部分構造による予測モデルの２種類のモデルを構築して予測精度の検討を行った．構造記述子モデルの構築には，The Chemistry Development Kit（CDK）[15] により基本的な構造記述子20個及びXlogP値を計算し，新規物質データを学習セットとしてWeka[16] を用いてRandomForest[17] による予測モデル構築を行い，既存物質データによる検証を行った．RandomForestは，決定木と同じ分類機の一種で，集団学習法と呼ばれ複数の決定木を作成し，これら組み合わせることで精度と汎化力を両立するモデル構築手法である．構造記述子やLogP値は，同一スケールの値ではないため，そのままの値を判別分析や重回帰分析に適用することは出来ない．一方，決定木であればデータへの制約はないが，あまり高精度の分類は期待出来ないため，今回の検討ではRandomForestを用いた．部分構造モデルでは，Molecular Substructure Miner（MOSS）[18] を用いて新規物質1231個を解析して肝毒性物質群に特徴的な部分構造フラグメント111個を抽出した．毒性予測はフラグメントに対する部分構造マッチングではなくフラグメントとの類似性距離（Tanimoto係数）をパラメータとしてRandomForestによる予測モデルを構築して既存物質データによる検証を行った．Table. 2にそれぞれのモデルの学習セット，検証セットでの予測成績を示す．学習セットに対する予測では，いずれのアプローチによってもほぼ100%の一致率を示すモデルが得られたが，検証セットに対する予測一致率はいずれも70%以下であった．得られたモデルの予測精度は良いとは言えないが，前項に示した市販ソフトにより構築したモデルと比べ遜色ない．（Q)SARを広く受け入れられる安全性評価手法として活用していくためには，なるべく多くの人々が安価に利用可能であることが望ましい．我々の研究は，必ずしも独自ツールの開発を目的としてはいないが，パブリックに入手可能なツールを用いて実用可能なモデル構築が可能であるなら，誰でも再現が可能である．もちろん，高精度の予測を行うために特定の市販ソフトウェアが必要不可欠であれば用いるべきである．いずれにせよ，当面の課題は，現時点で実現可能な予測レベルを認識した上で，信頼性向上に向けた検討を進めるとともに，まずは限定的であっても社会的に受用可能な利用法を提案していく中で徐々に適用範囲を広げていくことである．OECDでは，（Q)SARの行政的利用と受け入れの促進に向けた活動を進めており，行政目的で利用される（Q)SARモデルについてアルゴリズムや学習データの公

Table 2 Predictive performance of two different type SAR models for liver toxicity using publically available tools

	Training set		Test set	
No. of chemicals*	515/745		137/117	
Modeltype	Discriptor	Structure	Discriptor	Structure
Concordance	99.9	98.9	66.1	63.4
Sensitivity	99.8	98.1	67.2	62.0
Specificity	100.0	99.5	65.0	65.0

*Positive for liver toxicity/Negative for liver toxicity

開等５項目からなるOECD原則[19]を定めるとともに，加盟各国の提供により各国で蓄積されてきた毒性試験情報や（Q)SARモデル等を統合したOECD QSAR Toolboxの開発を進めている[20]．現在のバージョンのOECD QSAR Toolboxには，人に対する毒性評価モデルとして前述のCramer ruleが登録されている．

６．課題と将来展望

これまでの検討では，いずれの手法により構築した予測モデルにおいても*in vivo*肝毒性について精度の高い予測結果は得られていない．この原因として，学習セットと検証セットに含まれる化学構造の偏りや手法によって適応出来ない化学構造が含まれている可能性が挙げられる．適応化合物の制限や適応性の異なる複数のモデルを組み合わせて利用することでより高精度の予測結果が得られる可能性[11,21]はあるが，別の原因として生体側の反応に関する情報の不足が挙げられる．毒性が化学物質と生体との複雑な相互作用により発現する現象であるのに対して，構造活性相関では化学物質側の特性からの評価しか出来ない．つまり，生体側の情報が不足しているのである．受容体作用のようなターゲット（メカニズム）が特定されている反応であれば，３次元ドッキングのように反応側の情報を加味したアプローチも可能である．しかし，メカニズムやターゲットが解明されている毒性は非常に限られており，そうしたアプローチは利用出来ない．近年，（Q)SARと共に安全性評価の効率化の手段として期待されているのが*in vitro*評価系である．米国EPAが中心となって開始したToxCast（Tox21）プログラムでは，多数の*in vitro*ハイスループットアッセイと（Q)SARを組み合わせた毒性評価手法の構築を進めている[22]．毒性分野における*in vitro*評価系の多くは動物試験の代替法として開発される系が多いが，ToxCastにおける*in vitro*アッセイの多くは，創薬分野で用いられるメカニズムアッセイであり，個々のアッセイ結果は必ずしも直接毒性と結び付くものではなく，むしろ，毒性予測におけるパラメータとしての化学物質と生体の様々な相互作用反応を示すものと捉えることが出来る．一つの化学物質の安全性評価のために，ToxCastにおける*in vitro*アッセイを全て実施するのはコストや効率の面から現実的ではないが，数種の*in vitro*系により，化学構造からは計算出来ない重要なパラメータについてデータギャップを埋めることが可能であれば，（Q)SARと*in vitro*系を個別に用いるよりも，効率的かつ信頼出来る安全性評価ストラテジーが確立可能であろう．今後はそうした方向からの検討も進めていきたいと考えている．

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レギュラトリーサイエンスにおけるコンピュータを用いた構造活性予測研究の現状と展望

広瀬明彦

Researches on the *in silico* prediction of structure-activity relationship in the regulatory science sectors

Akihiko Hirose

Requirements of *in silico* toxicity prediction system are increasing in the chemical risk assessment fields, as well as in toxicity prediction at the early stage of the new drug development process. Recent amended chemical registration rules require internationally the risk assessment of huge amounts of existing chemicals. The (quantitative) structure-activity relationship ((Q)SAR) models are considered to be most effective tools for the acceleration of toxicity evaluation. In Europe or the United State, several research projects for the development of the (Q)SAR models are ongoing. Following this introduction, four researches on development of *in silico* prediction systems for (Q)SAR in the NIHS are reviewed. These activities must internationally contribute to the integrated chemical risk assessment approaches and/or could assist in the new drug development work.

Keywords: structure-activity relationship, in silico toxicity prediction, risk assessment

コンピュータを用いた（定量的）構造活性相関（(Q)SAR）モデルの発展は，近年のめざましいコンピュータ性能の進化と相俟って，大型コンピュータを必要とした複雑な計算を机上のパーソナルコンピュータで行うことを可能とすると共に，現実的でなかった生体分子の構造や医薬品などの化学物質との原子レベルでの相互作用を解析することが可能となるなど著しいものがある．これらコンピュータを用いた基盤的な（Q)SAR研究の進展は，基礎生物学的な生体反応の解明だけにとどまらず，応用的には特に創薬開発研究などへの貢献が期待されてきていた．一方，このような*in silico*技術は医薬品や環境化学物質と生体との相互作用により引き起こされる有害影響を説明することにも利用できることは明らかであり，近年，医薬品や化学物質の安全性評価を行う研究者や欧米の規制当局側においてリスク評価に有効的なツールとして利用するための試みが活発化してきている．

2007年より施行された欧州の化学品REACH規制（化学物質の登録，評価，許可，制限に関する規則）においては，それまでの既存化学物質や新規化学物質の区別を無くし，年間1トン以上製造または輸入される物質すべてについて登録が義務づけられ，製造・輸入量に応じて要求される毒性情報レベルは異なるものの，2018年までに約3万種といわれる既存化学物質の毒性情報を収集，評価することが求められている．しかし，数万種にも及ぶ化学物質すべてに対して要求される毒性試験を行うことは不可能であることは明らかであり，動物愛護の観点も考慮すると時間と費用を費やしてでも*in vivo*毒性試験を継続していくという選択も，社会的な理解を得ることは困難なところである．一方で，動物実験の代替法としての*in vitro*試験法を開発するための国際的な活動も近年活発化しているが，*in vivo*毒性試験よりスループットが高い*in vitro*試験をもってしても，数万種に及ぶ化合物の実測データを収集することは容易ではなく，しかも多様なエンドポイントをすべて代替するための*in vitro*試験の開発には，まだ相当の時間と技術の向上を必要としている段階である．そのため，類似構造に基づく共通の有害影響の可能性を推定することによるカテゴリーアプローチや（Q)SARモデルの適用は必須のものであると考えられている．欧州ではREACH規則への適用を目指した（Q)SAR研究プロジェクト（ToxTree[1],

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OpenTox[2], Caesar Project[3]）が進行しており，これらに市販のDEREKやMultiCASEなどのモデルも取り込んで，既存の毒性試験データベースを基にカテゴリー作成の支援を行うOECD QSAR application toolboxという統合化プラットフォームの開発もOECDのイニシアチブで進行している．米国では，大規模な*in vitro*試験データを基にした毒性予測システムの開発を目指したToxCastプロジェクト[4]などのcomputational toxicologyが進んでいるところである．本特論ではこのような国際的な動向に対応して，国立医薬品食品衛生研究所の4つの部を中心に行われている（Q)SAR研究を紹介する．これらは，現状ではまだ欧米プロジェクト等との直接的な連携は行われていないが，昨年の化審法改正にみられるレギュラトリー分野での国際化に向けて，今後レギュラトリー分野で最も注目される研究分野の一つとなることは疑う余地はない．

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Determination of a new designer drug, N-hydroxy-3,4-methylenedioxymethamphetamine and its metabolites in rats using ultra-performance liquid chromatography–tandem mass spectrometry

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ABSTRACT

An *N*-hydroxy analogue of 3,4-methylendioxymethamphetamine (MDMA), *N*-hydroxy MDMA (*N*-OH MDMA), has recently been distributed as a new designer drug in some drug markets. Very little data is available to the metabolic and pharmacological properties of *N*-OH MDMA, although it has been reported that the *N*-demethyl analogue, *N*-hydroxy-3,4-methylenedioxyamphetamine (*N*-OH MDA), is mainly metabolized to MDA in rats. In this study, an analytical method for the determination of *N*-OH MDMA and its metabolites in biological samples was developed, and the metabolic properties of *N*-OH MDMA in rats were investigated.

After the *i.p.* administration of *N*-OH MDMA to pigmented hairy rats (5 mg/kg/day, 10 days), *N*-OH MDMA and its *N*-dehydroxy and *N*-demethyl metabolites (MDMA, *N*-OH MDA and MDA) in rat plasma, urine and hair samples were determined by ultra-performance LC (UPLC)–MS/MS. The hair sample was extracted by 1-h sonication and overnight soaking in 5 M hydrochloric acid–methanol (1:20). The plasma, urine, and hair extract samples were purified using a solid-phase extraction procedure. *N*-OH MDMA in the samples could be precisely analyzed by avoiding an alkaline environment. The parent compound very rapidly disappeared from the rat plasma (<15 min) and urine (<10 h), and most of the *N*-OH MDMA was excreted in the rat urine as MDMA and MDA in 72 h. In the rat hair samples collected 4 weeks after the first administration, *N*-OH MDMA (0.03 ng/mg) and *N*-OH MDA (0.13 ng/mg) were clearly detected as well as MDMA (149 ng/mg) and MDA (52 ng/mg). This analytical method will be useful for the analysis of *N*-OH MDMA and its metabolites in biological samples.

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1. Introduction

Various designer drugs of 3,4-methylenedioxymethamphetamine (MDMA) have appeared as street drugs in recent years. Besides *N*-alkyl derivatives of MDMA such as 3,4-methylenedioxyethylamphetamine (MDEA), the use of beta-keto compounds such as methylone, 1-(3,4-methylenedioxyphenyl-2-(pyrrolidin-1-yl)-1-pentanone (MDPV), 2-methylamino-1-(3,4-methylenedioxyphenyl)butan-1-one (bk-MBDB), and 2-ethylamino-1-(3,4-methylenedioxyphenyl)propan-1-one) (bk-MDEA) has become widely spread throughout the world [1–7]. Moreover, an *N*-hydroxyl analogue of MDMA, *N*-hydroxy MDMA (*N*-OH MDMA, FLEA), has also been distributed as a new designer drug in some drug markets [5,8].

The *N*-hydroxy group has been found to have unique analytical properties in similar compounds such as *N*-hydroxy-3,4-methylenedioxyamphetamine (*N*-OH MDA; *N*-demethyl analogue of *N*-OH MDMA). *N*-OH MDA is unstable at high temperatures (e.g. GC–MS analysis) and decomposes to MDA and the oxime of 3,4-methylenedioxyphenyl-2-propanone [9]. Moreover, *N*-OH MDA (p*K*a value = 6.22) is considerably less basic than MDA (p*K*a value = 10.04), and thus its capacity factors show greater variation with mobile phase pHs in the 2.5–6.0 range for HPLC analysis, while retention of the primary amine, MDA, and *N*-alkyl MDAs remains relatively constant over this range [10]. The aqueous solution stability of *N*-OH MDA has been found to decrease with increases in the aqueous solution pH, and the degradation half-life decreases to a value of 2.57 h at pH 10 [10]. In an alkaline environment, *N*-OH MDA is mainly transformed to its corresponding oxime. This pH-dependent mechanism is different from that in the pyrolysis of *N*-OH MDA, as observed in the GC–MS analysis. On the other hand, Ravis et al. [11] have reported that *N*-OH MDA is rapidly metabolized to MDA in rats and that no other major metabolites could be detected in a rat liver slice, plasma, or urine sample. As compared with *N*-OH MDA, very little data is available as to the analytical, metabolic, and pharmacological properties of

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N-OH MDMA, although it has been reported that direct GC–MS analysis of N-OH MDMA gives no indication of the parent compound and that only MDMA (N-dehydroxy compound) and MDA (N-dehydroxy and N-demethyl compound) are detected as the major components of the sample [8].

In this study, the stability of N-OH MDMA in sample solutions under various conditions (including wide pH ranges) was studied to establish suitable conditions for animal studies. Furthermore, a rapid and sensitive analytical method for the simultaneous determination of N-OH MDMA and its metabolites in rat plasma, urine, and hair samples was developed using ultra-performance liquid chromatography–tandem mass spectrometry (UPLC–MS/MS), and the metabolic properties of N-OH MDMA in rats were investigated.

2. Materials and methods

2.1. Chemicals and reagents

MDMA hydrochloride [12], MDA hydrochloride [12], N-OH MDA hydrochloride [13] and 2-methylamino-1-phenylpropane-2,3,3,3-d4 hydrochloride (methamphetamine(MA)-d4, used as an internal standard) [14] were prepared, as previously reported. N-OH MDMA oxalate was synthesized from 3,4-methylenedioxyphenetylacetone and N-methylhydroxylamine according to the procedure reported by Noggle et al. [9]. Its structure and purity were confirmed by the measurements of accurate mass, the infrared spectrum [8], GC–MS (acetylated derivatives) [8], LC–MS [8], and ^{1}H- and ^{13}C-nuclear magnetic resonance (NMR) [5]. The accurate mass of $[M+H]^+$ was m/z 210.11309 in the positive scan mode by AccuTOF JMS-T100 (JEOL, Tokyo, Japan). The error between the observed mass and the theoretical mass of $[M+H]^+$ ($C_{11}H_{16}NO_3$) was +0.07 mmu. The structures of these drugs are shown in Fig. 1. A solid-phase extraction column (Bond Elut Plexa, 30 mg/1 mL) was obtained from Varian (Harbor City, CA, USA), and the membrane filter (Ultrafree-MC, 0.45 μm) was from Millipore Corporation (Bedford, MA, USA). All other chemicals and solvents were of an analytical reagent grade or HPLC grade (Wako Chemicals, Osaka, Japan).

2.2. Instrumentation

The UPLC analysis was performed using a Waters Acquity Ultra-Performance™ liquid chromatography system (Waters, Milford, MA, USA). The separations were achieved using an Acquity HSS T3 column (100 mm × 2.1 mm i.d., 1.8 μm) from Waters (Milford, MA, USA). The column temperature was maintained at 40 °C, and the following gradient system was used with a mobile phase A (1% formic acid) and mobile phase B (1% formic acid/acetonitrile) delivered at 0.3 mL/min: 90% A/10% B (0 min)–70% A/30% B (8 min). The mobile phase was used as a wash solvent to avoid any carry-over from previous injections. The auto-sampler was maintained at 4 °C and the injection volume was 2 μL. The total run time for each sample analysis was 8.0 min. Quantitation was achieved by MS/MS detection in a positive ion mode using a Quattro Premier XE mass spectrometer (Waters, Milford, MA, USA) equipped with an electrospray ionization (ESI) interface. Quantification was performed using multiple reaction-monitoring (MRM) of the transitions of m/z 210.2 → 163.2 for N-OH MDMA (4.5 min), m/z 196.2 → 163.2 for N-OH MDA (3.9 min), m/z 194.3 → 163.2 for MDMA (3.3 min), m/z 180.2 → 163.2 for MDA (2.9 min), and m/z 154.2 → 92.1 for IS (3.1 min), with a scan time of 0.05 s per transition. The cone voltage and collision energy were set at 20 and 15 for N-OH MDMA, MDMA, and IS, and at 20 and 10 for N-OH MDA and MDA, respectively. The optimal MS parameters obtained were as follows: capillary 3.0 kV, source temperature 120 °C, and desolvation temperature 400 °C. Nitrogen was used as the desolvation and cone gas, with a flow rate of 800 and 50 L/h, respectively. Argon was used as the collision gas, with a flow rate of 0.25 mL/min. All data collected in the centroid mode were processed using MassLynx™ NT4.1 software with a QuanLynx™ program (Waters, Milford, MA, USA).

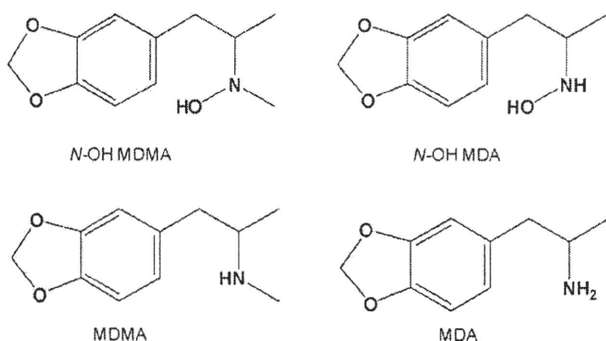

Fig. 1. Structures of N-OH MDMA and its metabolites.

2.3. Animal experiments

The animal experimental model was designed as shown in our previous reports [15,16]. All experiments were carried out with the approval of the Committee for Animal Care and Use of National Institute of Health Sciences, Japan. N-OH MDMA oxalate was administered to male dark agouti (DA) pigmented rats, which were 5 weeks old and around 90 g mean weight (Japan SLC, Shizuoka, Japan). The drugs were given once daily at 5 mg/kg by intraperitoneal injection for 10 successive days. Blood samples were collected 5, 15, 30, 60 120, and 360 min after the first administration from the orbital vein plexus. Plasma samples were prepared by centrifugation at 10,000 × g for 3 min and stored at −20 °C until analysis. The area under the plasma concentration time curve (AUC) was calculated by the conventional method [15]. Urine samples were collected 0–10, 10–24, 24–34, 34–48, and 48–72 h after the last administration and stored at −20 °C. To prevent the degradation of N-hydroxy compounds in the urine samples, 1 mL of 1 M phosphate buffer (pH 3) was added in advance to the collection vials and then cooled in an ice-bath (4 °C), and the pH of the collected urine was kept under acidic conditions. Each animal had been shaved on the back just before the first drug administration. The new growing hair samples were collected 28 days after the first administration.

2.4. Sample preparation

2.4.1. Stock solution

An individual standard solution of 1.0 mg/mL of each drug, N-OH MDMA, N-OH MDA, MDMA, and MDA, was prepared in methanol and stored at 4 °C. The IS solutions of 2 μg/mL of MA-d4 in methanol for the analysis of hair samples and those of 2 μg/mL of MA-d4 in distilled water for plasma and urine samples were also prepared.

2.4.2. Stability of N-OH MDMA

To investigate the stability of N-OH MDMA under various pH conditions, 0.5 mL of sample solutions containing 0.1 μg/mL of N-OH MDMA and MA-d4 (IS) were prepared with 0.1 M phosphate buffers at various pHs. The pHs of the buffer solutions, containing either mono- or di-basic potassium phosphate, were adjusted to 3.0, 4.0, 5.0, 6.0, 7.0, 8.0, 9.0, and 10.0 by adding 1 M phosphoric acid or 1 M potassium hydroxide, respectively. Fifty microliters of each sample solution was pipetted into the corresponding test tube, into which was previously added 0.45 mL of the mixed solution of methanol and acetonitrile (1:1) at 0, 1, 2, 4, 5, and 24 h after sample preparation, and analyzed using the UPLC–MS/MS system. The results were calculated using the peak-area ratios of the ions monitored for the target compounds versus IS, and indicated as a percentage of the remaining N-OH MDMA.

To establish suitable conditions for animal studies, N-OH MDMA and MA-d4 (IS) were dissolved in the rat drug-free urine (pH 8–9) at concentrations of 1 μg/mL. The stability of N-OH MDMA in the urine was studied under six different conditions described as follows: (1) kept at room temperature, (2) added 1 mL of 1 M phosphate buffer (pH 3.0) and kept at room temperature, (3) kept on ice, (4) added 1 mL of 1 M phosphate buffer (pH 3.0) and kept on ice, (5) the control urine was heated at 70 °C for 15 min before the addition of the drugs and was kept at room temperature, and (6) the urine was heated at 70 °C for 15 min and 1 mL of 1 M phosphate buffer (pH 3.0) was added before addition of the drugs. One hundred microliters of the sample under each condition was pipetted into the corresponding test tube, into which was previously added 1 mL of 0.1 M of phosphate buffer (pH 3.0) at 0, 1, 2, 4, 5, and 24 h after the sample preparation, and immediately the solution was treated with Bond Elut Plexa and analyzed as below. For the rat plasma samples, N-OH MDMA and MA-d4 (IS) were dissolved in the rat drug-free plasma (pH 7) at concentrations of 1 μg/mL. After being maintained at room temperature or on ice for 1, 2, and 4 h, 100 μL of each sample was pipetted and analyzed using the same method as with the urine samples.

2.4.3. Extraction of N-OH MDMA and its metabolites from plasma and urine samples

To a 50-μL plasma sample or a 100-μL urine sample were added 50 μL of the IS aqueous solution and 1 mL of 0.1 M phosphate buffer (pH 3.0), respectively. Before the quantitative analysis of MDMA and MDA, due to their high concentrations, the urine (0–10, 10–24, and 24–48 h) and plasma samples were diluted with the control specimens 100 times and 5 times as concentrations, respectively. After a Bond Elut Plexa was pre-activated with methanol and distilled water, the sample solution was applied to the Bond Elut Plexa. After the column was washed with 0.5 mL of distilled water, 0.5 mL of the solution of 2% formic acid/methanol was passed through the column to elute the target drugs. Following evaporation of the solvent under a nitrogen stream, the residue was dissolved in 0.5 mL of the mixed solution of methanol and acetonitrile (1:1). Two microliters of the solution was automatically injected into the UPLC–MS/MS.

2.4.4. Extraction of N-OH MDMA and its metabolites from hair samples

Hair samples were washed three times with distilled water under ultrasonication. After the sample was dried under a nitrogen stream at room temperature, approximately 10 mg of finely cut hair was precisely weighed and extracted with 1.5 mL of methanol/5 M hydrochloric acid mixed solution (20:1) containing 50 μL of each IS methanol solution for 1 h under ultrasonication. For the quantitative analysis of MDMA and MDA, 2-mg hair samples were used separately. Following overnight storage at room temperature, the hair was filtered off, the filtrate was evaporated with a nitrogen stream, and the residue was dissolved in 1 mL of 0.1 M phosphate buffer (pH 3.0). The solution was treated with Bond Elut Plexa and analyzed as above.

2.4.5. Linearity, precision, and recovery of the analytical method

The drug concentrations in the samples were calculated using the peak-area ratios of the ions monitored for the target compounds versus IS. The calibration curves for the determination were constructed by analyzing extracted drug-free control samples spiked with the standard solution, as described above. The calibration samples containing 0, 0.5, 1, 5, 10, 50, 100, and 500 ng/mL of the target drugs for the rat plasma and urine samples were prepared just before analysis. The samples containing 0, 0.01, 0.025, 0.05, 0.1, 0.25, 0.5, and 0.75 ng/mg of N-OH MDMA and N-OH MDA, and 0, 1, 2.5, 5, 10, 25, 50, and 75 ng/mg of MDMA and MDA for the hair samples were also prepared. For the urine analysis, 1 M phosphate buffer (pH 3.0) was added to the drug-free control urine samples before the addition of the standard solution of target drugs, to prevent the degradation of N-OH compounds. The limit of quantitation of each drug was chosen to be the concentration of the lowest calibration standard with an acceptable limit of variance.

The precision of the method was evaluated by five consecutive analyses of the plasma and urine samples that were spiked with the standard solutions containing 0.5, 50, and 500 ng/mL of the target drugs, respectively. For the hair analyses, the control samples, spiked with the standard solutions each containing 0.01, 0.05, and 0.5 ng/mg of N-OH MDMA and N-OH MDA and 1, 5, and 50 ng/mg of MDMA and MDA, were evaluated. The limit of detection (LOD) was defined as concentrations in a sample matrix resulting in peak areas with signal-to-noise (S/N) ratios of 3. The extraction recoveries of the four analytes, using the solid-phase extraction column, were determined using 0.1 M phosphate buffer (pH 3.0) spiked with the analytes at a concentration of 100 ng/mL, respectively. To determine the recoveries, the responses of the analytes spiked in the solutions before and after extraction were compared.

3. Results and discussion

3.1. Stability of N-OH MDMA

N-OH MDA, an N-demethyl analogue of N-OH MDMA, was unstable at high temperatures or in alkaline environments. It mainly decomposed to the oxime and/or its N-dehydroxy compound [9,10]. To evaluate the stability of N-OH MDMA oxalate in the stock solution, the methanol or aqueous solution of N-OH MDMA at a concentration of 1 mg/mL was kept at 4 °C for 2 weeks and the ratio of the remaining drug was measured. In both solutions, more than 90% of N-OH MDMA remained and thus they can be used as stock solutions for at least 2 weeks. In the same way, the stability of N-OH MDMA in the solution for the UPLC–MS/MS measurement was also studied. The ratios of the remaining drug of the solutions of 0.1% formic acid, acetonitrile, methanol, methanol/acetonitrile (1:1), 0.1% formic acid/10% acetonitrile (the initial composition of the mobile phase for the UPLC–MS/MS analysis) and 2% formic acid/methanol (the solution for eluting the drugs from the solid-phase extraction column) at a concentration of 0.1 μg/mL were analyzed over 24 h. As a result, the mixed solution of methanol/acetonitrile (1:1) was found to be the most suitable for the measurement of the UPLC–MS/MS system in this study.

To investigate the stability of N-OH MDMA under various pH conditions, the ratios of the remaining drugs in the buffer solutions under various pHs were analyzed over 24 h. N-OH MDMA was relatively stable in acidic conditions below pH 5, although it rapidly decomposed under basic conditions. Almost no parent compound was detected in the buffer solutions above pH 8 (Fig. 2). Beckett and Al-Sarraj [17] have reported that N-hydroxyamphetamine is readily decomposed into both the corresponding *syn*- and *anti*-oximes in alkaline solutions. The rate of the decomposition of N-hydroxyamphetamine is increased by dissolved oxygen in the solution, and a free radical mechanism has

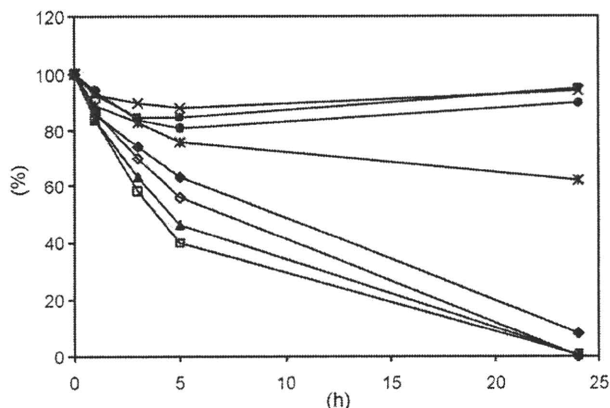

Fig. 2. Stabilities of N-OH MDMA oxalate in phosphate buffers at different pHs. ■, pH 3.0; ×, pH 4.0; ●, pH 5.0; *, pH 6.0; ◆, pH 7.0; ▲, pH 8.0; □, pH 9.0; ◇, pH 10.0.

been proposed [17]. In contrast, Valaer et al. [10] have reported that N-OH MDA is chemically decomposed to its oxime and that this mechanism is pH-dependent. In our additional study, we detected the oxime- and nitroso-compounds of MDA as the decomposed products of N-OH MDMA in alkaline environments by NMR analyses [18]. Under these conditions, as compared with N-OH MDA, some other mechanism would be responsible for the chemical transformation of N-OH MDMA to its corresponding N-demethyl oxime- and nitroso-compounds.

Because of the instability of N-OH MDMA in an alkaline environment, it appears likely that this drug would decompose in rat urine samples (pH 8–9). To set suitable conditions for animal studies, the ratios of the remaining drug in the rat control urine (pH 8–9) with added N-OH MDMA were analyzed under six different conditions over 24 h (Fig. 3). The ratios of the remaining N-OH MDMA of the urine samples kept on ice were approximately 80% after 4 h and 50% after 24 h, while no N-OH MDMA was detected in the urine kept at room temperature after 24 h. When the urine samples were heated before adding the drugs to remove the influences of bacteria and endogenous enzymes, the ratios of the remaining drug were almost the same as those of the non-treated urine samples and no N-OH MDMA was detected after 24 h at room temperature. Furthermore, when the pH of the urine samples was adjusted to pH 4–5 before adding the drugs, the ratios of the

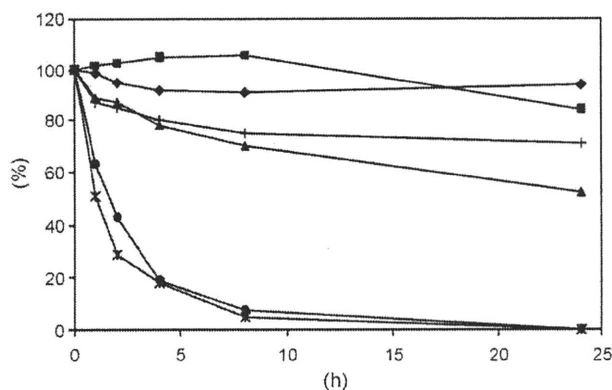

Fig. 3. Stabilities of N-OH MDMA oxalate in urine samples kept under different conditions. ●, kept at room temperature; ■, added 1 mL of 1 M phosphate buffer (pH 3.0) and kept at room temperature; ▲, kept on ice; ◆, added 1 mL of 1 M phosphate buffer (pH 3.0) and kept on ice; *, the control urine was heated at 70 °C for 15 min before addition of the drugs and was kept at room temperature, +, the urine was heated at 70 °C for 15 min and 1 mL of 1 M phosphate buffer (pH 3.0) was added before addition of the drugs.

Table 1
Linear ranges, calibration curves, and RSDs of analyses of N-OH MDMA and its metabolites in rat plasma, urine, and hair samples.

Compounds	Linear ranges	Calibration curves[a]	Conc. added	Conc. measured	RSDs (%)[b]
Plasma (ng/mL)					
N-OH MDMA	0.5–500		0.5	0.7	17
		$y = 0.5797x + 0.1111$	50.0	69.8	2.4
		$r^2 = 0.9988$	500.0	534.5	4.3
N-OH MDA	0.5–500		0.5	0.7	26
		$y = 0.4413x + 0.0647$	50.0	64.7	3.7
		$r^2 = 0.9983$	500.0	493.3	4.6
MDMA	0.5–500		0.5	0.3	7.1
		$y = 1.2243x + 0.2837$	50.0	58.5	2.3
		$r^2 = 0.9984$	500.0	513.2	2.3
MDA	0.5–500		0.5	0.4	17
		$y = 1.2047x + 0.2660$	50.0	61.8	1.8
		$r^2 = 0.9994$	500.0	530.9	1.8
Urine (ng/mL)					
N-OH MDMA	0.5–500		0.5	0.3	5.5
		$y = 0.8636x + 0.2218$	50.0	44.2	2.2
		$r^2 = 0.9981$	500.0	540.0	2.2
N-OH MDA	0.5–500		0.5	0.3	6.8
		$y = 0.3836x - 0.0111$	50.0	46.2	2.7
		$r^2 = 0.9983$	500.0	449.5	1.1
MDMA	0.5–500		0.5	0.3	6.9
		$y = 0.4741x + 0.2510$	50.0	53.0	2.5
		$r^2 = 0.9920$	500.0	522.7	16
MDA	0.5–500		0.5	0.3	27
		$y = 0.5269x + 0.2294$	50.0	45.2	4.9
		$r^2 = 0.9812$	500.0	465.3	2.1
Hair (ng/mg)					
N-OH MDMA	0.01–0.75		0.01	0.01	4.6
		$y = 4.8339 - 0.0019$	0.05	0.03	7.1
		$r^2 = 0.9900$	0.50	0.42	2.3
N-OH MDA	0.01–0.75		0.01	0.01	4.3
		$y = 2.3578 - 0.0056$	0.05	0.04	5.7
		$r^2 = 0.9892$	0.50	0.56	2.6
MDMA	1–50		1.0	1.2	2.5
		$y = 3.2766 + 0.8837$	5.0	5.2	2.4
		$r^2 = 0.9953$	50.0	53.1	1.5
MDA	1–50		1.0	1.1	2.5
		$y = 2.4426 + 0.2389$	5.0	5.5	1.9
		$r^2 = 0.9980$	50.0	55.3	1.2

[a] Weighting: $1/x$.
[b] RSD: relative standard deviation ($n = 5$).

Table 2
Time courses of drug concentrations in rat urine after the last administration of N-OH MDMA (5 mg/kg, *i.p.*).

Rat	Compounds	Concentrations in urine (μg/mL)				
		0–10 h	10–24 h	24–34 h	34–48 h	48–72 h
Rat 1	N-OH MDMA	0.05	TR	TR	TR	TR
	N-OH MDA	0.34	0.04	TR	TR	TR
	MDMA	75.3	12.4	1.97	0.68	0.17
	MDA	53.6	13.0	2.09	0.53	0.18
	Volume (mL)	2.5	5.3	2.8	3.6	6.2
Rat 2	N-OH MDMA	TR	TR	ND	ND	ND
	N-OH MDA	0.16	0.01	TR	TR	TR
	MDMA	63.4	7.35	1.79	0.73	0.36
	MDA	42.8	7.69	1.64	0.72	0.39
	Volume (mL)	2.7	4.3	3.0	3.6	6
Rat 3	N-OH MDMA	TR	TR	TR	ND	ND
	N-OH MDA	0.16	0.03	TR	TR	TR
	MDMA	69.9	12.5	3.43	0.64	0.66
	MDA	31.3	13.4	2.51	0.51	0.51
	Volume (mL)	3.5	3.8	3.0	2.8	3.7

TR: trace level, <10 ng/mL. ND: not detected.

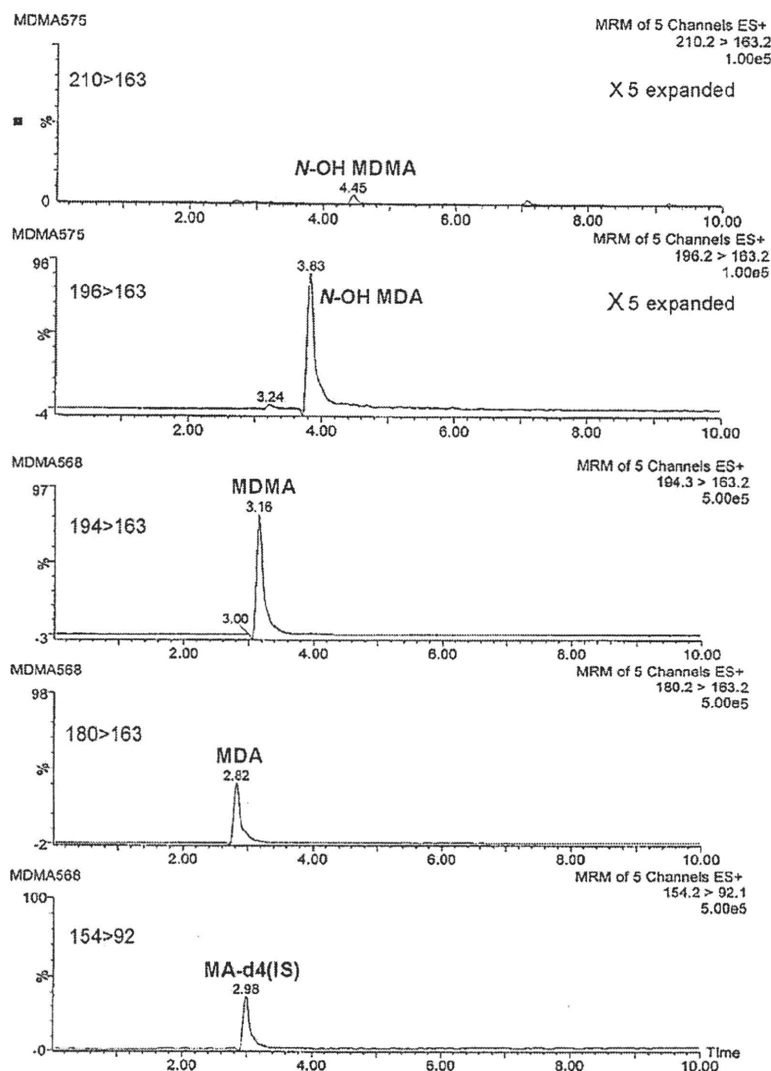

Fig. 4. UPLC–MS/MS MRM chromatograms of the extract from the rat urine 0–10 h after the last administration of N-OH MDMA (5 mg/kg, *i.p.*, rat 1).

remaining drugs after 24 h were approximately 95% on ice and 85% at room temperature (Fig. 3). On the basis of these results, adequate volumes of the phosphate buffer (pH 3.0) were added in advance to the collection vials in an ice-bath (4 °C), and the collected urine was kept under the acidic conditions in this study. On the other hand, no serious decomposition of N-OH MDMA in the plasma control samples kept on ice or at room temperature was observed for at least 2 h.

3.2. Pre-treatment steps

N-OH MDMA was unstable under the basic conditions, as described above. Moreover, it has been reported that N-OH MDA is considerably less basic than MDA and that its capacity factors show greater variation with mobile phase pH values for HPLC analysis using a C18 column [10]. Therefore, for the simultaneous extraction of target drugs from biological samples, a polymer-based solid-phase extraction column (Bond Elut Plexa™) was used without any basic solvents. According to the method described in Section 2, the recoveries of N-OH MDMA and its metabolites, N-OH MDA, MDMA, and MDA, from the samples added with their standard solutions (100 ng/mL), were 93.0, 85.5, 101.5 and 94.8%, respectively.

3.3. Linearity and precision of the analytical method for the rat urine, plasma, and hair samples

Under the chromatographic conditions used, there was no interference with any of the drugs or the internal standard by any extractable endogenous materials in the control rat plasma, urine, and hair extracts. The calibration curves were linear over the concentration range 0.5–500 ng/mL for rat plasma and urine, and 0.01–0.75 ng/mg (N-OH MDMA and N-OH MDA) and 1–50 ng/mg (MDMA and MDA) for rat hair with good correlation coefficients of $r^2 \geq 0.981$, respectively. The LODs of each drug were 0.1 ng/mL for the urine and plasma samples and 5 pg/mg for the hair samples, respectively. The precision data from the analytical procedure (n = 5) for the rat urine, plasma and hair samples, spiked with standard solution of N-OH MDMA, N-OH MDA, MDMA, and MDA, are presented in Table 1.

3.4. Time course of excretion of N-OH MDMA and its metabolites into rat urine

After intraperitoneal administration of N-OH MDMA oxalate to 3 rats at 5 mg/kg, the concentrations of N-OH MDMA and its metabolites in the rat urine were monitored using UPLC–MS/MS.

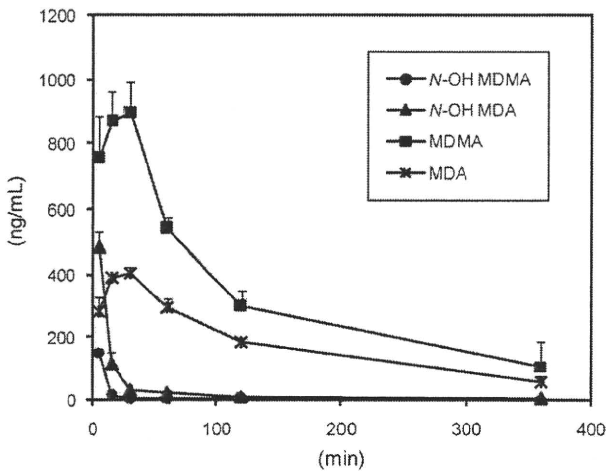

Fig. 5. Time courses of rat plasma drug concentrations after the first administration of N-OH MDMA (5 mg/kg, *i.p.*, *n* = 3). The error bar indicates each standard deviation.

The time courses of excretion of N-OH MDMA, N-OH MDA, MDMA, and MDA in the urine over 72 h are shown in Table 2. Fig. 4 shows LC–MS/MS MRM chromatograms of the extract from the rat urine 0–10 h after the last administration of N-OH MDMA (rat 1). The major metabolite excreted in the rat urine was MDMA (the N-dehydroxy metabolite), and 63–76 μg/mL of MDMA was detected from 0 to 10 h after administration. MDA (the N-dehydroxy and N-demethyl metabolite) was also detected at high concentrations of 31–54 μg/mL in the 0–10 h urine. In contrast, N-OH MDMA and N-OH MDA (the N-demethyl metabolite) were slightly detected only in 0–10 h urine and 0–24 h urine, and accounted for approximately 0.01 and 0.16% of the dose, respectively. More than 90% of the dose was excreted as MDMA and MDA in the rat urine in 72 h (Table 3), although other minor metabolites were not examined in this study. N-OH MDA has also been reported to be rapidly metabolized to MDA, and no other major metabolites have been detected in rats [11]. The rapid N-dehydroxylation of N-OH MDMA/N-OH MDA would make it difficult to discriminate N-OH MDMA/N-OH MDA use from MDMA/MDA use by urine analysis.

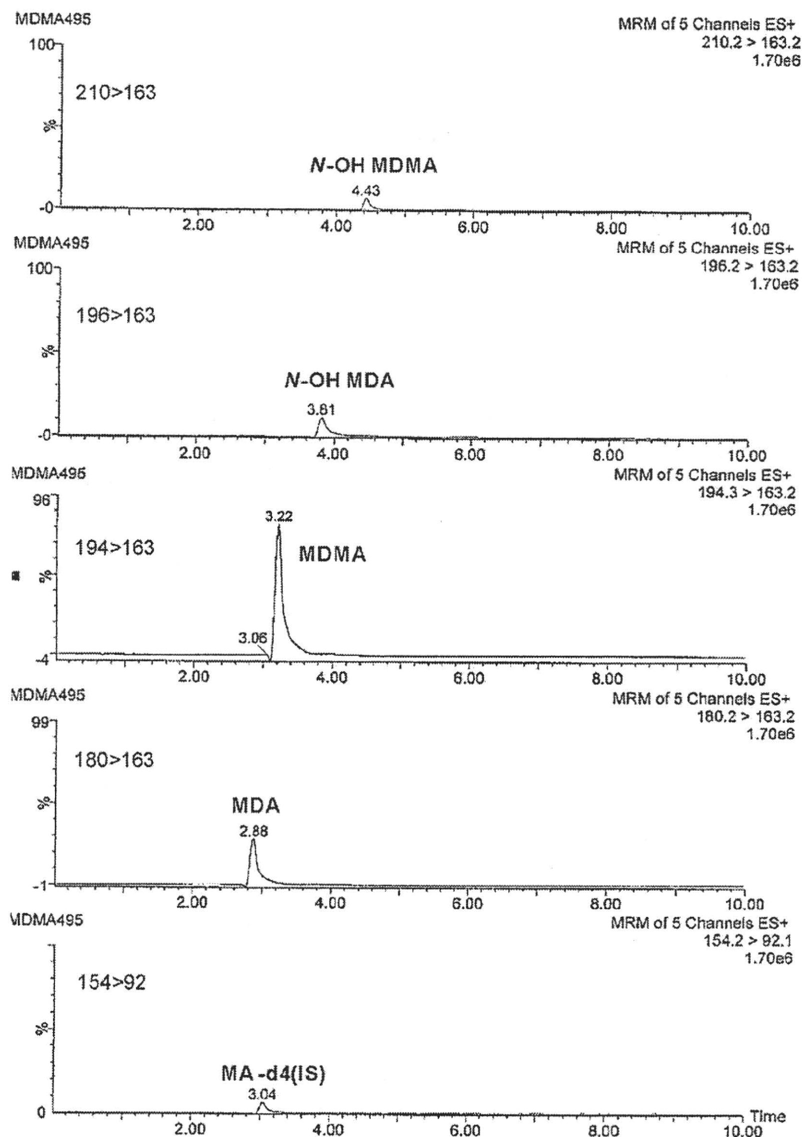

Fig. 6. UPLC–MS/MS MRM chromatograms of the extract from the rat plasma 5 min after the first administration of N-OH MDMA (5 mg/kg, *i.p.*, rat 1).

Table 3
The amounts of *N*-OH MDMA and its metabolites in urine, plasma, and hair.

Rat	Compounds	Total excretion into urine (μg, 0–72 h)	Plasma AUC (μg min/mL)	Concentrations in hair (ng/mg)
Rat 1	*N*-OH MDMA	0.1	1.1	0.03 ± 0.00
	N-OH MDA	1.1	7.2	0.07 ± 0.00
	MDMA	263.0	149.6	163.3 ± 17.3
	MDA	211.8	74.0	48.7 ± 0.7
Rat 2	*N*-OH MDMA	0.0	1.4	0.02 ± 0.00
	N-OH MDA	0.5	7.5	0.08 ± 0.00
	MDMA	212.9	130.9	117.6 ± 17.6
	MDA	158.8	77.6	41.4 ± 1.7
Rat 3	*N*-OH MDMA	0.0	1.4	0.03 ± 0.006
	N-OH MDA	0.7	10.2	0.23 ± 0.02
	MDMA	306.7	143.2	164.5 ± 11.1
	MDA	171.5	89.0	66.3 ± 1.4

3.5. Concentrations of N-OH MDMA and its metabolites in rat plasma

Fig. 5 shows the time courses of the rat plasma concentrations of *N*-OH MDMA and its metabolites over 360 min after the first administration of *N*-OH MDMA oxalate at 5 mg/kg. LC–MS/MS MRM chromatograms of the extract from the rat plasma 5 min after the administration of *N*-OH MDMA (Rat 1) are shown in Fig. 6. The concentrations of *N*-OH MDMA and *N*-OH MDA were

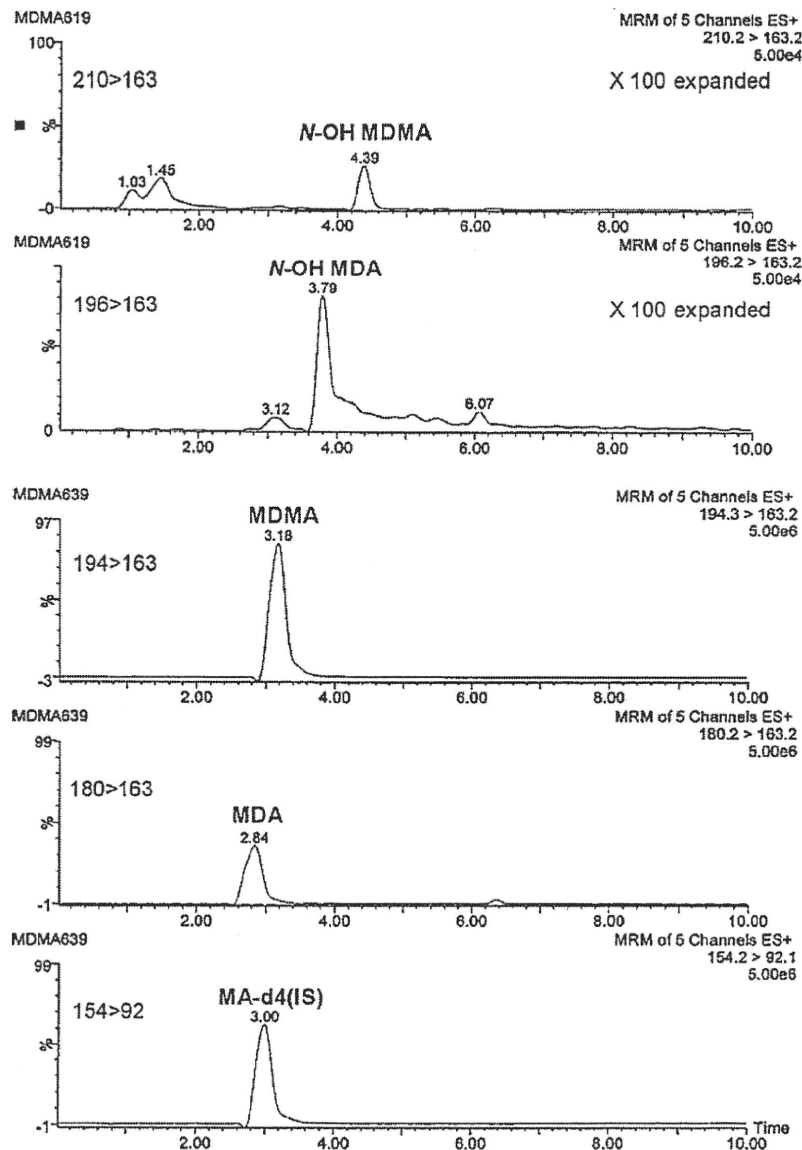

Fig. 7. UPLC–MS/MS MRM chromatograms of the extract from the rat hair collected 4 weeks after the first administration of *N*-OH MDMA (5 mg/kg × 10 days, *i.p.*, rat 1).

extremely low and their average peak concentrations ($n = 3$) were 130 ng/mg at 5 min and 490 ng/mL at 5 min, respectively. It was difficult to detect N-OH MDMA in the plasma at 120 min after administration. The concentrations of the major metabolites, MDMA and MDA in the plasma showed peaks (970 and 410 ng/mL) within 30 min. The AUC values of N-OH MDMA, N-OH MDA, MDMA, and MDA in the rat plasma were 1.1–1.4, 7.2–10.2, 130.9–149.6, and 74.0–89.0 μg min/mL, respectively, as shown in Table 3. The AUC values of MDMA and MDA were approximately 110 and 65 times larger than those of N-OH MDMA, respectively.

3.6. Drug concentrations in rat hair

Various procedures for the extraction of drugs from hair samples have been reported, including digestion with alkali, acid extraction, and enzymatic treatment [19,20]. Because N-OH MDMA is unstable under alkaline conditions, the procedures using alkali digestion (above pH 10) and enzymatic treatment (above pH 7) may not be acceptable for the extraction of N-OH MDMA. We have reported that the mixed solution of methanol and 5 M hydrochloric acid (20:1) is suitable for the extraction of phenethylamine-type compounds from hair samples [20,16]. Therefore, in this study, the acidic organic solvent was used for the extraction of N-OH MDMA and its metabolites from the rat hair. To investigate the stability of these drugs during the extraction procedure described in Section 2, the rat control hair samples, with added N-OH MDMA and its metabolites (10 ng/mg each), were analyzed. As a result of the analysis, almost no degradation of the N-OH compounds was observed.

Fig. 7 shows UPLC–MS/MS MRM chromatograms of the extract from the rat hair collected 4 weeks after the first administration of N-OH MDMA (5 mg/kg × 10 days, *i.p.*, rat 1). In the rat hair samples, although MDMA (149 ng/mg) and MDA (52 ng/mg) were mainly detected in large quantities, N-OH MDMA (0.03 ng/mg) and N-OH MDA (0.13 ng/mg) were also clearly detected. The detection of N-OH compounds from the hair samples might provide useful information for distinguishing N-OH MDMA use from MDMA use over a long period. However, it has been reported that N-OH MDMA and N-OH MDA are also detectable as N-hydroxylated metabolites in the urine of horses, orally administered with MDMA [21]. It may therefore be difficult to conclude whether N-OH MDMA detected in biological samples is the parent compound or the N-hydroxylated metabolite of MDMA. In further studies, the ratios of the parent compound to the metabolites in the samples obtained from N-OH MDMA users and MDMA users should be examined to deduce the source of the compound detected.

4. Conclusions

In this study, we have established a detailed procedure for the analysis of N-OH MDMA, N-OH MDA, MDMA, and MDA in rat urine, plasma, and hair samples using UPLC–MS/MS. Moreover, the established method was applied to investigate the metabolic properties of N-OH MDMA in rats. N-OH MDMA in biological samples could be precisely analyzed by avoiding alkaline environments. N-OH MDMA very rapidly disappeared from rat plasma and urine, and most of the N-OH MDMA was excreted in rat urine as MDMA and MDA in 72 h. The rapid N-dehydroxylation of N-OH MDMA would make it difficult to discriminate N-OH MDMA use from MDMA use by urine analysis. In the rat hair samples collected 4 weeks after the first administration, N-OH MDMA and N-OH MDA were clearly detected as well as MDMA and MDA, which were found to be the major metabolites in hair. The proposed analytical method will be useful for the analysis of N-OH MDMA and its metabolites in biological samples.

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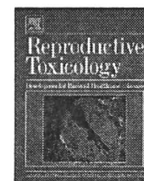

Prenatal developmental toxicity of gavage or feeding doses of 2-*sec*-butyl-4,6-dinitrophenol in rats

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ABSTRACT

This study evaluated the prenatal developmental toxicity of the pesticide 2-*sec*-butyl-4,6-dinitrophenol (dinoseb). Pregnant rats were given dinoseb by gavage at 0, 8.0 or 10 mg/kg bw/day on days 6–15 of gestation, or in the diet at 0, 120 or 200 ppm (0, 6.52 or 8.50 mg/kg bw/day) on days 6–16 of gestation, and litters were evaluated on day 20 of gestation. Maternal toxicity was observed as evidenced by significantly decreased body weight gain and reduced food consumption during the administration period in all the dinoseb-treated groups, and two dams died at 10 mg/kg bw/day. Significantly lower fetal weights and delayed skeletal ossification was observed in the dinoseb-treated groups except for the group fed dinoseb at 120 ppm. The teratogenic potential of the gavage dose of dinoseb was confirmed as evidenced by increased incidences of fetuses with external and skeletal malformations at 10 mg/kg bw/day. The incidence of fetuses with microphthalmia was significantly increased at this dose. On the other hand, feeding doses of dinoseb up to 200 ppm did not induce teratogenicity in this study. These data indicate that dinoseb is teratogenic at maternally toxic doses, but the exposure range of dinoseb at which malformations occur seems to be narrow.

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1. Introduction

Dinoseb, 2-*sec*-butyl-4,6-dinitrophenol (CAS No. 88-85-7) was approved for sale in the US in 1948 as a nitrophenolic herbicide in soybeans, vegetables, fruits, nuts, citrus and other field crops for the selective control of grass and broadleaf weeds [1,2]. Dinoseb is also used as an insecticide for grapes and as a seed crop-drying agent [2]. Dinoseb is one of the chemicals permitted on the market on the basis of safety tests conducted by Industrial Bio-Test Laboratory, a concern later found to have submitted many flawed and even fraudulent reports on its procedures and results [3]. Subsequently, several studies showed that dinoseb has the potential to produce developmental toxicity including teratogenicity in rats, mice and rabbits [4–7].

Dinoseb as a pesticide was banned in the US in 1986 and the EU in 1991 owing to the potential risk of adverse health effects in humans [2,8], but dinoseb and its salts are still widely used as other agricultural products [9]. Dinoseb is a high volume chemical with production or importation exceeding 1000 tons/year in Organisation for Economic Co-operation and Development (OECD) member countries [10]. Dinoseb as a pesticide is also banned in Japan but its import is permitted [9], and the volumes of dinoseb imported into Japan were estimated to be 827 tons in fiscal year 2007 and 615 tons in fiscal year 2008 [11].

Exposure to dinoseb may occur by direct contact, ingestion or inhalation by users and producers. Indirect exposure to dinoseb via the environment is also anticipated. The microbial breakdown of dinoseb has been demonstrated in soils, but dinoseb persists for about 2–4 weeks after application [12]. A soil persistence of 24–42 months was also observed in potato fields in Canada [13]. It has been reported that dinoseb was detected in water supplies in Canada and the US, and dinoseb residues were found in a cotton meal sample [12].

In previous review papers, we showed that dinoseb possesses testicular toxicity [14] and developmental toxicity [15] in experimental animals. We reported the results of a combined repeated dose toxicity study with a reproduction/developmental toxicity screening test, in which Crj:CD(SD)IGS rats were administered dinoseb by gavage at 0, 0.78, 2.33 or 7.0 mg/kg bw/day. At 7.0 mg/kg bw/day, eight females died and two animals were moribund during late pregnancy, and a significant decrease in body weight gain was found in both sexes. The numbers of dams that delivered their pups and dams with live pups at delivery were significantly reduced at this dose. Because only two females in the highest dose group delivered their pups, the developmental toxicity of dinoseb was not fully assessed in this study [16], but gross internal and external examinations revealed no significant differences in the incidence of pups with malformations. In a previous review

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[15], we concluded that teratogenic susceptibility to dinoseb was greater in rabbits than in rats and mice. Several studies failed to demonstrate the teratogenicity of dinoseb in rats [16–18], but we consider that the teratogenic potential of dinoseb in rats is unclear for various reasons. The feeding dose of dinoseb to rats on days 5–14 of gestation increased the incidence of fetuses with microphthalmia at 200 ppm (15 mg/kg bw/day), but this was not observed by gavage dosing at 15 mg/kg bw/day [4]. The incidence of fetuses with microphthalmia also increased when dinoseb was given in a certain composition of diet (protein 21%, fat 4.8%, fiber 4.2%, ash 8.5% and N-free extractives 61.5%) at 200 ppm or by gavage with the same diet at 15 mg/kg bw/day on days 5–13 of gestation, but this effect was not observed when a different diet (protein 21%, fat 3.5%, fiber 6.5%, ash 7.5% and N-free extractives 61.5%) was fed to pregnant rats [19]. As described above, adequate experimental conditions for the production of fetal malformations by the administration of dinoseb to pregnant rats remain unknown. Therefore, the present study was conducted to clarify the experimental conditions that produce fetal malformations when dinoseb is given to pregnant rats.

2. Materials and methods

2.1. Animals

This study was performed in 2008 at the Safety Research Institute for Chemical Compounds Co., Ltd. (Sapporo, Japan). The study was conducted in accordance with "Act on Welfare and Management of Animals" [Act No. 105, October 1, 1973, revised December 22, 1999, Revised Act No. 221; revised June 22, 2005, Revised Act No. 68], "Standards Relating to the Care, Management and Refinement of Laboratory Animals" [Notification No. 88 of the Ministry of the Environment, Japan, April 28, 2006] and "Basic Policies for the Conduct of Animal Experiments in Research Institutions under the Jurisdiction of the Ministry of Health, Labour and Welfare" [Notification No. 0601005 of the Health Sciences Division, Ministry of Health, Labour and Welfare, Japan, June 1, 2006].

Male and female SPF Crl:CD (SD) rats were used throughout this study. This strain was chosen because it is most commonly used in toxicity studies, and historical control data are available. Rats at 8 weeks of age were purchased from Atsugi Breeding Center, Charles River Laboratories Japan, Inc. (Yokohama, Japan) and were quarantined and acclimated to the laboratory for 3 weeks prior to the start of the experiment. Male and female rats found to be in good health were selected for use. The animals were reared on a sterilized basal diet (CRF-1; protein 22%, fat 5.7%, fiber 2.9%, ash 6.3% and N-free extractives 55.3%; Oriental Yeast Co., Ltd., Tokyo, Japan) and tap water *ad libitum*. After the quarantine and acclimation, male and female rats were housed individually except during the mating period. Daily vaginal lavage samples of each female were evaluated for estrous cyclicity. Females showing proestrous vaginal smears were paired overnight with males on a 1:1 basis. The females were examined next morning for vaginal plugs and sperm in vaginal smears. The day on which the presence of sperm in the vaginal smear and/or a vaginal plug was detected was designated day 0 of gestation. The mated females were separated into three groups to equalize the female body weights in the gavage dose groups or the feeding dose groups. The animals were maintained in an air-conditioned room at a room temperature of 22 ± 3 °C, a relative humidity of 50 ± 20%, a 12-hour light/dark cycle and 10–15 air changes per hour.

2.2. Chemicals and dosing

Dinoseb was obtained from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). The dinoseb (Lot No. 010608LB-AC) used in this study was 100% pure, and was stored under refrigeration prior to use. The purity and stability of the chemical were verified by analysis before the study. Dose levels were determined on the basis of the results of studies by Giavini et al. [4,19]. At these doses, maternal and/or developmental toxicity was/were expected to be observed in the dinoseb-treated groups. For the gavage dose groups, 12 females per group were given dinoseb once daily by gastric intubation at 0 (control), 8.0 and 10 mg/kg bw from day 6 to day 15 of gestation. The dinoseb was suspended in corn oil, and the control rats were given only corn oil. The volume of each dose was adjusted to 5 ml/kg body weight on the basis of the latest body weight. The dosing suspensions were prepared once per 7 days, and were stored in the dark and cold conditions before use. For the feeding dose groups, 12 females per group were given dinoseb in the diet from day 6 to day 16 of gestation at 0 (control), 120 and 200 ppm, and were thus expected to consume similar amounts of dinoseb to those in the gavage groups. The control rats were given the basal diet. The diet for the dose groups was prepared more than once every 4 days and was stored at room temperature before use.

2.3. Observations

All female rats were observed for clinical signs of toxicity once a day before and after the administration period, twice a day during the administration period and once on the day of sacrifice. Body weight was recorded once a day during the administration period and on days 0, 18 and 20 of gestation, and body weight gain was calculated. Food consumption was recorded on days 0, 6, 9, 12, 16, 18 and 20 of pregnancy. Rats that died during the administration period were autopsied and grossly examined. The pregnant rats were killed by exsanguination under ether anesthesia on day 20 of gestation. The organs and tissues were grossly examined. The ovary and uterus were removed from the maternal body, and gravid uterine weight was recorded. The numbers of corpora lutea, implantation sites, live and dead fetuses and resorptions were recorded. The placenta was removed and weighed. The live fetuses were removed from the uterus, sexed, weighed and inspected for external malformations and malformations within the oral cavity. The live fetuses were put down using an intraperitoneal injection of a sodium pentobarbital solution, and the eyes of the fetuses were examined after the removal of the skin of the head. Then, approximately one-half of the live fetuses in each litter were fixed in Bouin's solution for the examination of internal anomalies. Their heads were subjected to free-hand razor-blade sectioning [20], and the thoracic areas were subjected to microdissection [21]. The remaining live fetuses in each litter were fixed in 70% ethanol, stained with Alizarin red S and alician blue, and examined for skeletal anomalies.

2.4. Data analysis

Maternal body weight gain, gravid uterine weight, food consumption, number of corpora lutea, number of implantations, number of live fetuses, number of dead or resorbed embryos/fetuses, fetal weight, placental weight and degree of ossification were analyzed for statistical significance as follows. Bartlett's test of homogeneity of variance was used to determine if the groups had equivalent variances at the 5% level of significance. If the variances were equivalent, the groups were compared by one-way analysis of variance. If significant differences were found, Dunnett's multiple comparison test was performed. If the group variances were not equivalent, the Kruskal–Wallis test was used to assess the overall effects. Whenever significant differences were noted, pair-wise comparisons were made using the Mann–Whitney *U*-test. Fetal weight, placental weight and degree of ossification were analyzed using the litter as a unit. Implantation index, viability index of fetuses, total incidence of dead or resorbed embryos/fetuses, incidence of fetuses with malformations or variations and sex ratio of live fetuses were analyzed by Wilcoxon's rank sum test using the litter as a unit. The 0.05 level of probability was used as the criterion for significance.

3. Results

Table 1 shows the maternal findings in rats given dinoseb by gavage or in the diet. At 10 mg/kg bw/day, death occurred on days 10 and 13 of gestation in one female each. No changes in clinical findings were observed in the feeding dose and the other gavage dose groups. Maternal body weight gain was significantly decreased on days 6–16 and 0–20 of gestation in all the dinoseb-treated groups and significantly increased on days 16–20 of gestation at 200 ppm. Food consumption was significantly decreased in the gavage dose groups on days 6–9 and 9–12 of gestation at both 8.0 and 10 mg/kg bw/day. After the administration period, food consumption was increased at 8.0 and 10 mg/kg bw/day, and a significant increase was observed on days 16–18 of gestation at 8.0 mg/kg bw/day. Similarly, food consumption was significantly decreased during the administration period in the feeding dose groups at 120 and 200 ppm, and it was significantly increased at 200 ppm after the administration period. The average intakes of dinoseb at 120 and 200 ppm were 6.52 and 8.50 mg/kg bw/day, respectively. At autopsy, dilatation of renal pelvis was observed in only one rat at 8.0 mg/kg bw/day, which was suggested to be spontaneous occurrence. Two animals that died during the administration period at 10 mg/kg bw/day showed abnormal findings such as discoloration of the lung and spleen, atrophy of the thymus, thickening limiting ridge of the stomach and/or dark red patch in the glandular stomach. No changes were observed in the feeding dose groups at autopsy (data not shown).

Table 2 presents the reproductive findings in rats given dinoseb by gavage or in the diet. Body weights of live fetuses were decreased in the dinoseb-treated groups, and significantly decreased body weights were noted in male fetuses at 10 mg/kg bw/day, in

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Table 1
Maternal findings in rats given dinoseb by gavage or in the diet.

Dose	Gavage dose			Feeding dose		
	Control	8.0 mg/kg	10 mg/kg	Control	120 ppm	200 ppm
No. of pregnant rats	12	12	12	12	12	12
Initial body weight	263.3 ± 10.4[a]	263.7 ± 10.0	262.8 ± 11.0	299.1 ± 21.8	298.9 ± 22.9	298.9 ± 25.9
No. of females showing clinical signs of toxicity						
Death	0	0	2	0	0	0
Body weight gain during pregnancy (g)						
Days 0–6	42.3 ± 7.9	36.8 ± 5.7	39.6 ± 5.4	27.1 ± 5.8	26.3 ± 7.4	24.2 ± 6.4
Days 6–16	59.3 ± 9.5	31.3 ± 7.4**	25.6 ± 8.2** (10)	48.7 ± 12.9	25.3 ± 5.2**	−11.4 ± 5.8**
Days 16–20	67.1 ± 8.4	70.8 ± 9.8	68.8 ± 9.9 (10)	64.1 ± 9.9	64.3 ± 9.8	81.4 ± 15.1**
Days 0–20	168.8 ± 18.4	138.9 ± 12.4**	133.3 ± 14.7** (10)	139.8 ± 20.1	115.9 ± 14.8**	94.2 ± 19.9**
Food consumption during pregnancy (g/day)						
Days 0–6	23.4 ± 1.8	22.9 ± 1.6	23.3 ± 1.4	21.5 ± 2.1	22.2 ± 2.5	20.4 ± 1.9
Days 6–9	21.0 ± 1.9	17.1 ± 1.4**	16.2 ± 2.4**	21.1 ± 2.3	16.8 ± 0.9**	12.0 ± 1.1**
Days 9–12	22.3 ± 2.2	19.7 ± 1.7*	19.5 ± 2.8** (11)	21.8 ± 4.2	17.2 ± 1.4**	11.7 ± 1.5**
Days 12–16	21.5 ± 2.1	20.5 ± 1.1	22.1 ± 1.9 (10)	22.4 ± 2.4	20.6 ± 3.2	15.6 ± 2.0**
Days 16–18	25.5 ± 2.2	28.2 ± 2.1**	27.6 ± 1.9 (10)	24.0 ± 2.5	25.2 ± 2.6	28.2 ± 2.9**
Days 18–20	26.3 ± 1.5	27.9 ± 2.6	28.0 ± 1.6 (10)	23.1 ± 2.6	24.2 ± 2.6	27.3 ± 2.8**

Values in parentheses are the number of animals examined.
[a] Values are given as the mean ± SD.
* Significantly different from the control ($p < 0.05$).
** Significantly different from the control ($p < 0.01$).

female fetuses at 8.0 and 10 mg/kg bw/day and in both sexes at 200 ppm. Weight of the placenta was significantly decreased at 10 mg/kg bw/day, but it was not affected by the feeding dose of dinoseb. Gravid uterine weight was decreased dose-dependently. No effects were observed in other reproductive parameters.

The summarized results of external and internal examinations of fetuses are shown in Table 3. External malformations were found in 1 out of the 171 fetuses (1 out of 12 litters) at 8.0 mg/kg and 18 out of the 147 fetuses (4 out of the 10 litters) at 10 mg/kg bw/day, and the incidence of fetuses with external malformations was significantly increased at 10 mg/kg bw/day. Among the fetuses at 10 mg/kg bw/day, there were 1 each with cleft palate or filamentous tail, 2 each with runt, anotia, brachymelia or ectrodactyly and 17 fetuses with microphthalmia. The incidence of fetuses with microphthalmia was significantly increased at this dose. No significant differences were found upon external examinations of the feeding dose groups. Runt was observed in one fetus at 8 mg/kg bw/day and each one fetus in two different litters at 10 mg/kg bw/day. In the internal examinations, no significant differences were observed in the gavage and feeding dose groups.

The summarized results of skeletal examinations of the fetuses are presented in Table 4. There were no significant differences between the dinoseb-treated and control groups in the incidence

Table 2
Reproductive findings in rats given dinoseb by gavage or in the diet.

Dose	Gavage dose			Feeding dose		
	Control	8.0 mg/kg	10 mg/kg	Control	120 ppm	200 ppm
No. of litters	12	12	10	12	12	12
No. of corpora lutea per litter	16.3 ± 2.3[a]	16.0 ± 2.2	15.9 ± 1.7	15.5 ± 1.6	15.4 ± 1.0	13.9 ± 2.9
No. of implantations per litter	14.9 ± 3.4	14.8 ± 2.5	15.2 ± 2.2	15.2 ± 1.9	14.4 ± 1.1	13.6 ± 3.0
Implantation index (%)[b]	90.5 ± 14.8	92.6 ± 12.5	95.4 ± 6.7	97.8 ± 4.4	93.6 ± 5.8	97.5 ± 3.7
Dead or resorbed embryos and fetuses						
Early stage[c]	0.4 ± 0.5	0.5 ± 0.5	0.4 ± 0.5	0.8 ± 0.7	0.7 ± 0.8	0.8 ± 1.3
Late stage[d]	0.0 ± 0.0	0.0 ± 0.0	0.1 ± 0.3	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
Total incidence (%)[e]	2.7 ± 3.4	3.1 ± 3.2	3.4 ± 3.7	5.4 ± 4.4	4.7 ± 5.5	6.6 ± 9.0
No. of live fetuses	14.5 ± 3.4	14.3 ± 2.1	14.7 ± 2.3	14.3 ± 1.7	13.8 ± 1.4	12.8 ± 3.2
Viability index of fetuses (%)[f]	97.3 ± 3.4	96.9 ± 3.2	96.6 ± 3.7	94.6 ± 4.4	95.4 ± 5.5	93.4 ± 9.0
Sex ratio of live fetuses[g]	0.472 ± 0.152	0.472 ± 0.136	0.447 ± 0.163	0.503 ± 0.133	0.506 ± 0.141	0.427 ± 0.152
Body weight of live fetuses (g)						
Male	4.043 ± 0.283	3.792 ± 0.285	3.425 ± 0.279**	4.033 ± 0.293	3.858 ± 0.281	3.620 ± 0.217**
Female	3.873 ± 0.228	3.587 ± 0.221*	3.240 ± 0.315**	3.780 ± 0.288	3.641 ± 0.253	3.399 ± 0.261**
Gravid uterine weight (g)	84.3 ± 19.1	78.9 ± 11.2	74.7 ± 11.5	84.1 ± 12.7	77.5 ± 8.5	70.1 ± 18.4*
Placental weight (g)	0.483 ± 0.047	0.467 ± 0.030	0.435 ± 0.046*	0.502 ± 0.045	0.477 ± 0.037	0.518 ± 0.096

[a] Values are given as the mean ± SD.
[b] (Number of implantations/number of corpora lutea) × 100.
[c] Includes implantation sites and placental remnants.
[d] Includes macerated fetuses and dead term fetuses.
[e] (Number of dead or resorbed embryos and fetuses/number of implantations) × 100.
[f] (Number of live fetuses/number of implantations) × 100.
[g] Number of live male fetuses/number of live fetuses.
* Significantly different from the control ($p < 0.05$).
** Significantly different from the control ($p < 0.01$).