ん試料については樹脂状と粉末状の試料を、薬包紙に少量とりイオン源にかざして測定した。その結果、主要なあへんアルカロイドであるモルヒネ、コデイン、テバイン、パパベリン及びノスカピンの $[M+H]^+$ に相当するイオンが検出され、測定値よりプロトン付加体の組成式が推定可能であった。液体クロマトグラフィーで定量を行うと、あへん試料中のモルヒネ含量は11～13％程度と高いにもかかわらず、本研究におけるDART測定条件では、モルヒネに対応するピークのイオン強度は低かった。そこで、同濃度に調製したあへんアルカロイド5化合物のメタノール溶液をガラス棒に付着させてDART-TOFMSで測定したところ、溶液状態においてもモルヒネのイオン強度が最も低い結果となった。したがって、あへん試料をDARTで分析する際は、モルヒネよりノスカピン等の他成分を指標とするのが望ましいと考えられた。なお、他に、あへん以外のけし由来試料として、一般に焼き菓子等食品に使用されているけしの実一粒について、DART-TOFMSにより測定したところ、含有成分であるモルヒネとコデインが検出されたとの報告もある[37]。

通常、大麻やマジックマッシュルーム、あへん中の麻薬成分を検出するためには、抽出操作が不可欠である。しかし、DART-TOFMSでは、前処理を加えることなく、植物片や樹脂そのものの形態で成分のイオン化が可能であり、精密質量値より化合物の組成推定を行うことにより含有成分の推定が可能である。DART-TOFMSは、法律で厳しく規制されるこれら植物についても、活性成分の簡易スクリーニング分析法として有用であると思われた。

4. いわゆる健康食品中に違法に添加された医薬品成分の分析への応用[5)]

近年、多くの種類の強壮を標榜する“健康食品”がインターネット経由で、容易に入手可能となった。その中には、実際に、性機能改善薬（ED治療薬）として日本においても処方せん医薬品として認可されているシルデナフィルやバルデナフィル、タダラフィル（図6）、またそれら医薬品の構造の一部を改変した構造類似化合物やPDE5阻害剤として開発された化合物（医薬品としては無認可）が、強壮作用を強める目的で違法に加えられている製品があり、検出例が続々と報告されている[38]。これら医薬品はいずれも、生体内のホスホジエステラーゼ（PDE）5をターゲットとし、本酵素を阻害することで、陰茎の海綿体の平滑筋の弛緩を引き起こし、勃起不全を改善する。医薬品の添付文書によると、これらは、硝酸剤あるいは一酸化窒素供与剤（ニトログリセリン、亜硝酸アミル、硝酸イソソルビド等）との併用により降圧作用が増強し、過度に血圧を下降させる恐れがある。また、死亡例

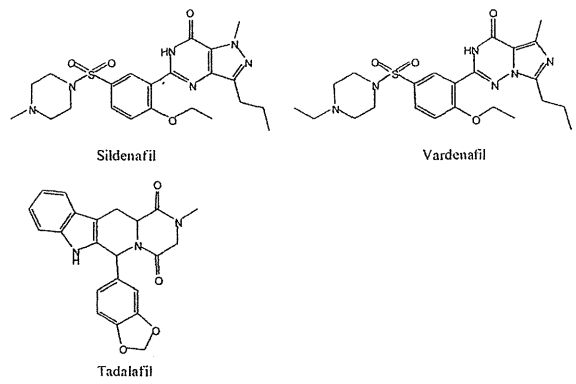

図6. ED治療薬の構造

を含む心筋梗塞等の重篤な心血管系等の有害事象が報告されている。さらに、構造類似体の中には、PDE5だけではなく、網膜に分布するPDE6に対しても同様に阻害活性を有するものもあり、失明等の重い副作用が起こる可能性も否定できない[39]。このような“健康食品”による健康被害を未然に防止するためには継続的に製品を買い上げ、成分分析調査を実施していく必要がある。

2007年度（平成19）までの5年間、強壮を標榜して市場に流通していたいわゆる健康食品661製品について、ED治療薬3成分及びPDE5阻害活性を有する構造類似化合物11成分を分析対象として、DART-TOFMSによるスクリーニング分析及びLC-MSによる一斉分析を行った。製品形態は、錠剤、カプセル、液体、粉末と様々であるが、DART-TOFMSでは特別な前処理を行わず（カプセルについては内容物を分析）、試料を直接イオン源にかざし分析を行った。また、LC-MSでは、1％ギ酸水溶液・アセトニトリル（1：4）溶液で超音波下抽出し、遠心分離、膜ろ過を行った試料について分析を行った。LC-MS分析の結果、661製品中112製品から分析対象成分が検出され、112製品の内17製品では複数の分析対象成分を含有していた。DART-TOFMS分析では、LC-MSで分析対象成分が検出された112製品のうち、他製品と比較して極端に低濃度しか検出されなかった2製品を除き、いずれも分析対象成分の $[M+H]^+$ に対応するピークが検出された。なお、DART-TOFMSにおいて検出が困難であった2製品は、HPLCのUV検出器でも分析対象成分は検出されなかった。これら“健康食品”は、HPLCやLC-MS等通常の分離分析手段で測定を行う際にはまずは目的成分の抽出操作を行う必要があるが、製品形態が様々であるため、特殊なマトリックスを有する製品では個別の抽出法を別途検討しなくてはいけない場合もある。また、分析対象成分とは異なる物性を有する違法成分が添加されていた場合、抽出時に十分回収できるとは限らず、含有を見落とす可能性も考えられる。DART-MS分析は、抽出操作なしで短時間に含有成分を直接測定でき、このような製品のスクリーニング法として有用であると考えられた。

5. おわりに

DARTは2005年（平成17）に発表された新しいイオン化手法であるが[1]、現在までに、食品化学、天然物化学、生化学、香料等化粧品化学、製剤、薬物動態、裁判化学、有機合成、工業原料等、様々な分野で応用例が報告されている。周辺機器の開発・改良も進み、2009年（平成21）になってDART-MS分析に関する報告数は急増している。

しかし、一方で、いくつかの欠点も指摘されている。DARTは、TLCで分離されたプレート上のスポットについては直接イオン化が可能であるが、その他のクロマトグラフィー等の分離手段とは組み合わせることが難しい。そのため、前処理を行わない場合、マトリックス中に大量に存在する他成分によるイオン化の妨害も否定できない。我々の経験では、尿中薬物分析において、適切な前処理を行わないと常在成分である尿素によるイオン化抑制がおき、目的化合物の検出感度が極めて低くなった。また、同一の元素組成を有する化合物は識別できない。さらに、エレクトロスプレーイオン化法と比較して、グルクロン酸抱合のような弱い結合を開裂する傾向にある[21]。モルヒネの主代謝物であるグルクロン酸抱合体についても、DART-MS分析では抱合体のプロトン化分子イオンは検出されずモルヒネとして検出されるため、薬物動態を検討する際に、未変化体を過剰に見積もる恐れがある[21]。我々の経験でも、プロトン化分子イオンが検出されやすい化合物とされにくい化合物が認められており、たとえば、DART-MS測定では、薬事法で指定薬物として規制されている亜硝酸エステル類は検出されなかった。さらに、DARTは分子量1000以上の化合物についてはイオン化が困難となる。検出感度の点でも、Petucciらの報告[18]では、ポジティブ、ネガティブ両モードにおいて、20種類の様々な構造を有する薬物を対象に、DARTとエレクトロスプレーイオン化法で得られるイオン強度を比較した結果、DARTは2～10倍低い値を示している。

DARTはイオン源と質量検出器の間が解放されており、大気圧下、試料をその部位にかざすだけで表面がイオン化される。そのため、固体、液体、気体を問わず、そのままの形態で迅速に直接分析が行えることが最大の特徴といえる。まだ改善の余地がある技術であるが、特殊なマトリックス内の特定成分の簡易迅速スクリーニング分析やリアルタイムモニタリング分析、また、TLCと組み合わせた分析で最も能力を発揮すると考えられる。どのような試料の何をターゲットとして分析するか、研究者の工夫次第で適用範囲が広がる可能性がある。

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PROFILE

花尻（木倉） 瑠理

国立医薬品食品衛生研究所生薬部
第3室室長
薬学博士

1990年千葉大学大学院薬学専攻博士課程前期修了、同年国立医薬品食品衛生研究所（旧国立衛生研究所）薬品部研究員、主任研究官を経て2002年生薬部第3室室長、現在に至る。この間2000年7月から2001年8月まで科学技術庁長期在外派遣研究員（米国カンザス大学）。

Bull. Natl. Inst. Health Sci., **128**, 29-33 (2010) **Special Report**

1）コンピュータシミュレーションを用いた薬物設計および違法薬物等の活性予測

栗原正明

Computational study on drug design and prediction of bioactivity for regulation of non-controlled psychotropic substances

Masaaki Kurihara

We demonstrated design and synthesis of rescue ligands for agonists of the mutant vitamin D receptor (Arg274Leu), and performed QSAR study of non-controlled psychotropic substances and docking study of non-approved or unauthorized pharmaceuticals, inhibitors of human phosphodiesterase 5 (PDE5) using computer simulation.

Keywords: drug design, mutant vitamin D receptor (Arg274Leu), QSAR, non-controlled psychotropic substances, non-approved or unauthorized pharmaceuticals

はじめに

ここでは2つのトピックについて書く．ひとつはコンピュータシミュレーションによる薬物設計であり，もう一つはQSAR（定量的構造活性相関）等を用いた違法薬物の規制についてである．共通のキーワードは「予測」である．薬物を設計するためには，設計した分子が「どのような構造をとるか？」，「タンパク質とどのような相互作用をするか？」を予測しなくてはならない．信頼できる予測ができないと，複雑な分子の合成に踏み込む勇気がわかない．薬物設計の例としては変異ビタミンD受容体救済型リガンドの設計に絞って述べる．一方，違法な薬物を迅速に規制するためにはQSARやドッキングスタディによる活性予測は有効である．動物試験や生物学的試験を行うことが最善であるが，それには多くの時間が必要となる．ここでは違法な薬物の活性予測についての実際の例を述べる．

1．変異ビタミンD受容体救済型リガンドの設計

1－1　2位α置換活性型ビタミンD_3誘導体リガンド

近年，癌の分子標的薬等の様にタンパク質の変異によって引き起こされる様々な疾病に対する薬物の開発が注目されている．核内レセプターの変異によって起こる疾病があり，これらの疾病は遺伝子の変異によって起きるもので，難病とされているものが多い．ビタミンDレセプター（VDR）の変異（Arg274Leu）により遺伝性ビタミンD抵抗性くる病が発症する．

ビタミンDレセプターは核内レセプターのひとつで，骨代謝，免疫調節等の作用を転写レベルで制御している．そのリガンドは活性型ビタミンD_3（1α,25$(OH)_2D_3$）である．（Fig. 1）VDRと活性型ビタミンD_3の複合体は4つの水素結合を形成していることが明らかになっている．（Fig. 2）

その中でもリガンドの1α-ヒドロキシ基とArg274のグアジノ基間での水素結合が特に重要である．変異（Arg274Leu）によりこの水素結合ができずリガンドはレセプターに結合しにくくなり，遺伝性ビタミンD抵抗性くる病が発症する．それを改善するために別の位置で水素結合を形成するようリガンドを設計すれば失われた

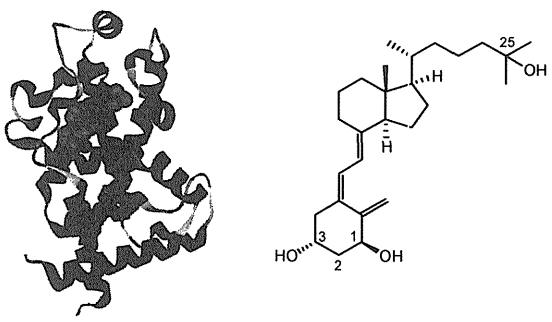


Fig. 1　VDR-LBD and 1α,25$(OH)_2D_3$

To whom correspondence should be addressed:
Masaaki Kurihara: 1-18-1 Kamiyoga, Setagaya-ku, Tokyo 158-8501, Japan; Tel: +81-3-3700-1141; Fax: +81-3-3707-6950; E-mail: masaaki@nihs.go.jp

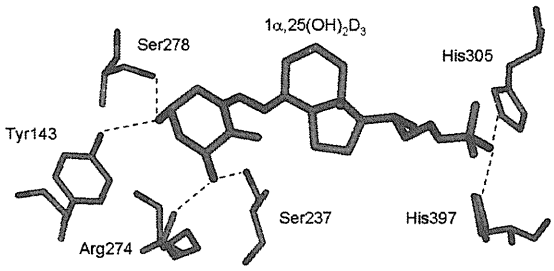


Fig. 2　1α,25(OH)$_2$D$_3$ bound to VDR

水素結合を補うことができると考えた．そのためにA環の２位に3-ヒドロキシプロポキシ基を導入した．(Fig. 3)

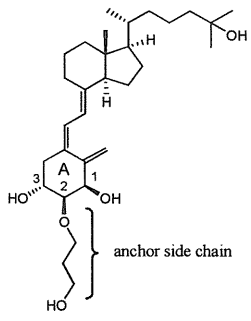


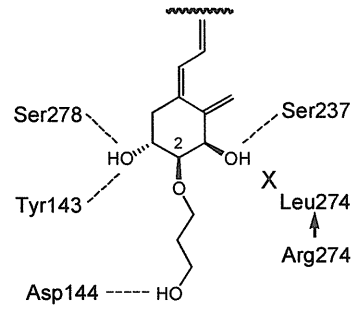
Fig. 3　2α-(3-Hydroxypropoxy)-1α,25-vitamin D$_3$

これはモデリングにより3-ヒドロキシプロポキシ基の末端水酸基がAsp144と水素結合することを予測したからである．(Fig. 4) タンパク質内のリガンドのコンフォメーションは，プログラム*MacroModel* (Schrodinger, Inc.) のコンフォメーショナルサーチ（条件：Mixed MCMM/Low Mode, Amber*）を用いて求めた．コンフォメーショナルサーチで得られた最安定構造を結合モデルとした．実際このリガンドは変異VDR (Arg274Leu) に対して活性型ビタミンDよりはるかに強く結合することが明らかとなった[1]．これをはじめとして幾つかのリガンドを設計，合成した[2, 3]．いずれも，変異によって失われた相互作用を補うように分子設計することにより結合を強める救済型のリガンドである．

ちなみに，２位α置換活性型ビタミンD$_3$誘導体は正常なVDRにも強い活性を持ったリガンドとなる[3]．

１－２　ノンセコステロイド型リガンド

著者等はセコステロイド骨格(ビタミンDの基本骨格)を持たなリガンドの創製（設計と合成）を行っている．すでにYR301 (Fig. 5) が活性型ビタミンD$_3$に匹敵する転写活性があることを見いだした[4]．さらにVDR-LBDとYR301のX線構造解析にも成功した[5]．その結合様式



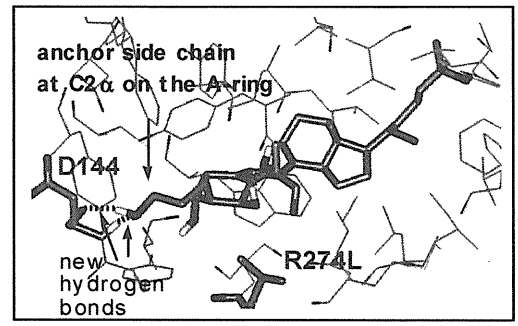


Fig. 4　Modeling structure of the rescue ligand bound to mutant VDR (Arg274Leu)

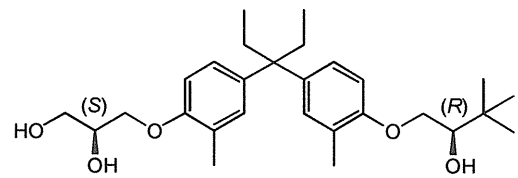


Fig. 5　Structure of YR301

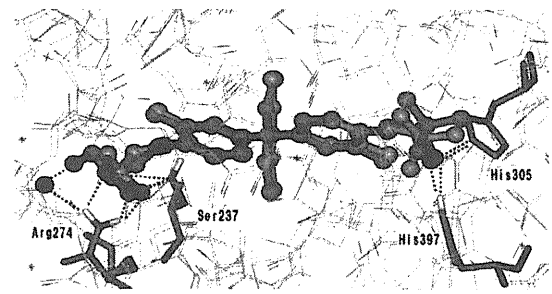


Fig. 6　Structure of YR301 bound to VDR-LBD. (dark: x-ray structure, light: modeled structrue)

は事前に予測したモデリングの構造 (Fig. 6) とよく一致した．

このノンセコ型のリガンドが救済型のリガンドにもなることを見いだした[6]．YR301の立体異性体であるYR303 (Fig. 7) と変位VDR (Arg274Leu) のモデリングの結果YR303の左側の末端の水酸基はSer237と水素結合することが予測された．(Fig. 8) 変異VDRを用いて

転写活性を測定したところではYR303は活性型ビタミンD_3よりも26倍の活性を示した．正常なVDRにおいてはArg274と水素結合している末端水酸基がSer237と水素結合することにより失われた水素結合を補填していると考えられる．これは鎖状構造の柔軟性によるものである．

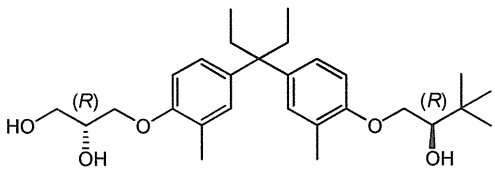


Fig. 7　Structure of YR303

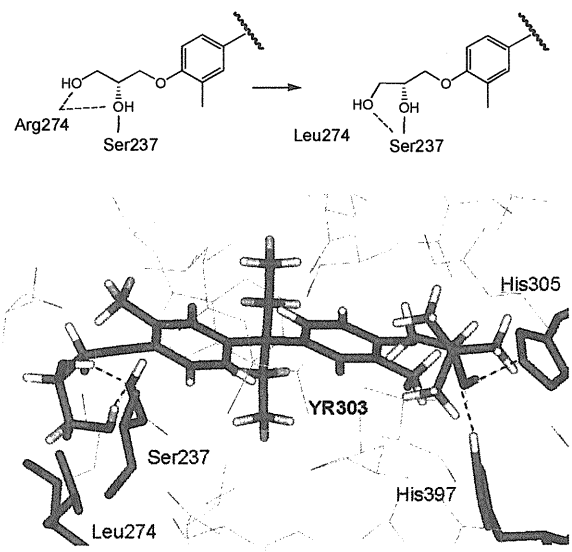


Fig. 8　Modeling structure of YR303 bound to mutant VDR (Arg274Leu)

ここで示した救済型リガンドの方法論は他の変異タンパクにも適用が可能である．変異により引き起こされる疾病は難病が多い．患者の数が少ない難病においては，治療薬の開発は製薬メーカーが行いにくい現状もあり，我々が取り組むべきひとつの課題であると考えている．

2．違法薬物等の活性予測

2－1　QSARを用いた違法薬物の活性予測

違法ドラッグが大きな社会問題となっている．そこで，違法ドラッグを速やかに規制するためには，違法ドラッグの迅速な評価法が必要である．動物実験や生物学的試験には多くの時間が必要であり，迅速な規制のためにはコンピュータを用いたインシリコ評価法を開発することが必要である．ここでは実際に行ったひとつの例として4-メチルメトカチノン（Fig. 9）の場合を述べる．

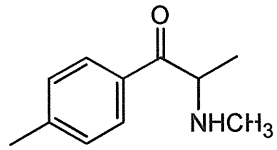


Fig. 9　Methcathinone

活性予測は2つの方法で行った．ファーマコフォアフィンガープリント法と2D-QSAR（定量的活性相関）である．

いずれも化学計算パッケージMOE（CCG社）を用いた．活性が既知の化合物として1～10の化合物を用いた．(Table 1) 活性値は(+)-アンフェタミン（1 mg/kg）で弁別したラットを用いて般化試験を行った際の構造類似化合物1～10の実測活性値（ED_{50}値）を採用し，それぞれの方法で4-メチルメトカチノン(11)の活性値を予測した．

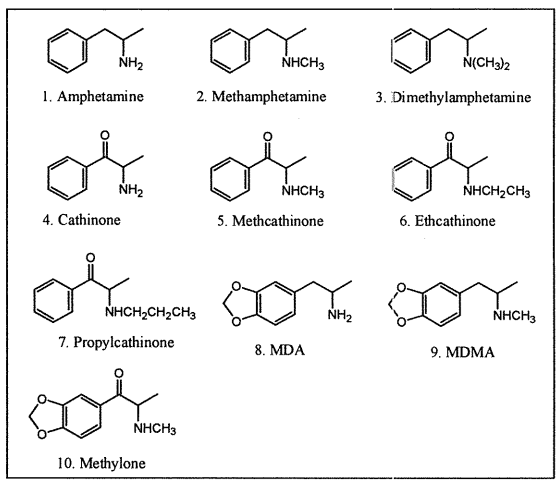


Fig. 10

Table 1　Psychotropic substances

No	Psychotropic substances	Activities* (ED_{50}, mg/kg)
1	Amphetamine	0.71
2	(±)-Methamphetamine	0.49
3	(+)-Dimethylamphetamine	2.92
4	(±)-Catinone	0.71
5	(±)-Methcatinone	0.37
6	Ethcathinone	0.77
7	Propylcathinone	2.03
8	MDA	2.29
9	MDMA	1.64
10	Methylone	2.36

* T. A. Dal Cason et al., Pharmacology Biochemistry and Behavior, 58(4) (1997) 1109-1116.

① ファーマコフォアフィンガープリント法

化学構造の類似性のみによる評価法として，ファーマコフォアフィンガープリント法による評価を行った．2点のファーマコファアのグラフ距離で分子の類似性を評価するTGD法を用いた．活性の強い化合物２，５をテンプレートとして構造の類似性を算定した．化学構造の類似性と活性値との相関から4-メチルメトカチノンの活性値を予測した．（Fig. 11，X軸：構造の類似性，Y軸：活性値）

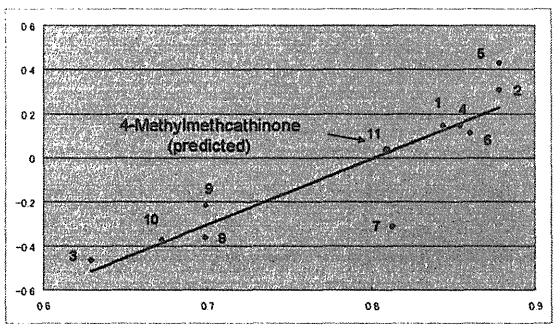


Fig. 11

② 2D-QSAR（定量的活性相関）法

AutoQuaSAR法を使って妥当なQSARモデル式を構築し，4-メチルメトカチノンの活性値を予測した．QSARモデル式で用いた記述子は，MOE上で動作するAutoQuaSARプログラムによって，MOEに搭載されている184のすべての２D記述子から選択されたものである．QSAR式は交差検定のR^2（相関係数の２乗）が最も良いものを用いた．（Fig. 12，X軸：活性値の予測，Y軸：活性値）

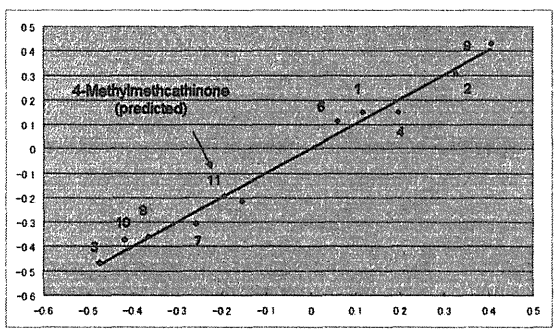


Fig. 12

相関係数の二乗（R^2）=0.84
交差検定の相関係数の二乗（XR^2）=0.92
QSAR式
pIC50 = 1.40145
　　−0.0365513 * PEOE_VSA-0
　　−0.0848188 * PEOE_VSA-1
　　−0.0168429 * SMR_VSA2
　　+0.0168429 * SlogP_VSA5

麻薬，覚せい剤，指定薬物を含む構造類似化合物10化合物の活性（既知）と比較するために，ファーマコフォアフィンガープリント法及び2D-QSAR（定量的活性相関）法の２方法で評価した．4-メチルメトカチノンは，どちらの方法でもこれら規制化合物群と同程度の活性があることが予測された．このデータは4-メチルメトカチノンを規制するための科学的データとして使われた．

２−２　シルデナフィル類似化合物の活性予測

無承認無許可医薬品であるシルデナフィル類似物質（PDE5阻害剤）が健康被害の危険性があり，大きな問題となっている．これら無承認無許可医薬品を迅速に規制，検挙するためには，これら不適切な化合物の迅速な活性評価スクリーニングが必要である．しかし，*in vitro, in vivo*試験では時間がかかり，迅速な対応が困難である．そこで*in silico*による評価法について検討した．

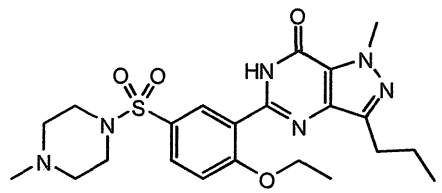


Fig. 13　Structure of Sildenafil

ここでは，ヒドロキシチオホモシルデナフィル（Fig. 14）について述べる．

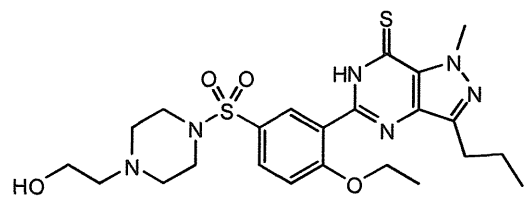


Fig. 14　Structure of hydroxythiohomosildenafil

シルデナフィルは，生体内で環状グアノシン一リン酸（cGMP）の分解を行っている５型ホスホジエステラーゼ（PDE5）の酵素活性を阻害する．これがNO作動性神経に作用して血管を拡張させ，血流量が増えることによって活性を発現すると考えられている．シルデナフィル類似物質とHuman Phosphodiesterase 5（PDE5）と

の結合モデルを以下のようにして構築した．PDE5の三次元構造はX線構造（PDB ID：1UDT）を用いた．タンパク質内の薬物のコンフォメーションは，プログラム *MacroModel*（Schrodinger, Inc.）のコンフォメーショナルサーチ（条件：Mixed MCMM/Low Mode, Amber*）を用いて求めた，コンフォメーショナルサーチで得られた最安定構造を結合モデルとした．水素結合を中心に結合モデルの評価を行った．ヒドロキシチオホモシルデナフィルとPDE5（1UDT）の結合モデルを構築した．（Fig. 15）その結果，これらはシルデナフィルが結合した部位にほぼ同一の配向性で結合することが明らかとなった．さらに，シルデナフィルは活性ドメインにおいてGln817と水素結合を形成するが，これらも同様にGln817と水素結合を形成しうることが示された．（Fig. 15）このことにより，これらは同一の作用機序でシルデナフィルの標的分子であるPDE5を阻害することが予測される．このデータはヒドロキシチオホモシルデナフィルを規制するための科学的データとして使われた．

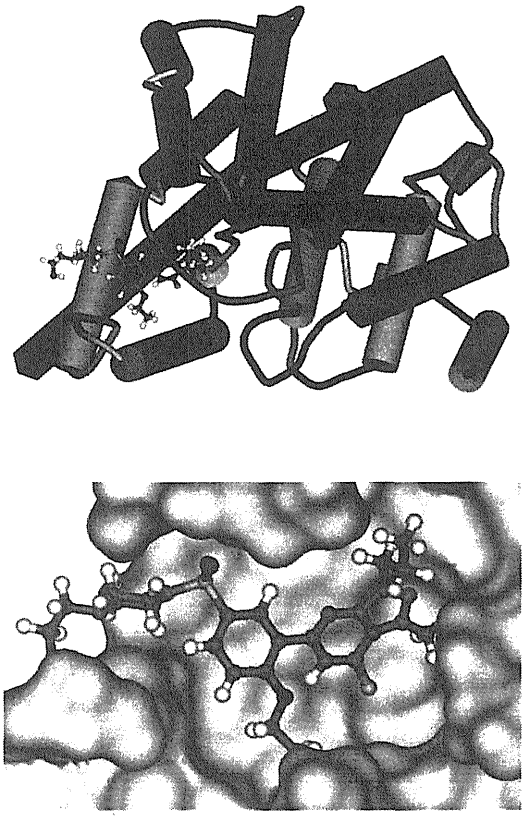


Fig. 15 Modeled structure of hydroxythiohomosildenafil bound to PDE5

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Simultaneous and group determination methods for designated substances by HPLC with multi-channel electrochemical detection and their application to real samples

Jun Zhe Min,[a]* Kazuhide Yamashita,[a] Toshimasa Toyo'oka,[a]* Shinsuke Inagaki,[a] Tatsuya Higashi,[a] Ruri Kikura-Hanajiri[b] and Yukihiro Goda[b]

ABSTRACT: Many psychotropic substances are illegally available on the streets and/or via the Internet. This wide distribution has become a serious social problem. To control this problem, many substances have been controlled as 'designated substances' (Shitei-Yakubutsu) in Japan since April 2007 by the Pharmaceutical Affairs Law, including tryptamines, phenethylamines and piperazines. In the present study, simultaneous determination methods using HPLC with multi-channel electrochemical detection (MECD) were developed for the designated substances. The proposed methods utilizing online electrochemical oxidation are the first report on the simultaneous determination of various designated substances. The methods involve direct determination and require no complicated pretreatments such as fluorescence labeling. The designated substances were separated by reversed-phase chromatography using a TSK-gel ODS-100V (4.6 × 250 mm, i.d., 3 μm) and gradient elution by a mixture of potassium phosphate buffer, methanol and acetonitrile. The total separation of 31 designated substances was successfully performed but required long chromatographic run times. Thus, the designated substances were divided into three groups: (1) tryptamines, (2) phenethylamines and (3) piperazines and others. They were then analyzed by HPLC-MECD as another separation method. The suitable applied voltages for each designated substance were determined based upon the hydrodynamic voltammogram. The limits of detection (signal-to-noise ratio of 3) of the designated substances for the most suitable voltages were in the range of 17.1 pg (5-MeO-MIPT) to 117 ng (indan-2-amine). The calibration curves based on the peak heights were linearly related to the amounts of the designated substances ($R^2 > 0.999$). Good accuracy and precision by intra-day assay and inter-day assay were also obtained using the present procedures. The proposed methods were applied to the analyses of the designated substance in several real samples. Copyright © 2010 John Wiley & Sons, Ltd.

Keywords: designated substances (Shitei-Yakubutsu); tryptamines; piperazines; phenethylamines; HPLC; multi-channel electrochemical detection

Introduction

Many narcotic substances are strictly controlled by the Narcotics and Psychotropic Control Law in Japan. However, various new analogs of these substances possessing phenethylamine, tryptamine and piperazine structures have appeared one after another in the drug market and have become one of the most serious social problems in Japan (Tanaka *et al.*, 2006; Yamamoto, 2004; Nicholos, 1981; Glennon and Rosecrans, 1982; Spoerke and Hall 1990; Jacob III and Shulgin 1994; Marek and Aghajanian, 1998; Kanai *et al.*, 2008; Nagai *et al.*, 2007). Principally, they should be controlled as non-authorized pharmaceuticals by the Pharmaceutical Affairs Law in Japan. However, a time-consuming procedure is needed to control them when these compounds are not specifically mentioned in the name list. For countermeasures against these substances, the Ministry of Health, Labor and Welfare amended the Pharmaceutical Affairs Law with a strengthened penalty provision in 2006. Thirty-one non-controlled psychotropic substances (11 tryptamines, 11 phenethylamines, six alkyl nitrites, two piperazines and salvinorin A) and one plant

* Correspondence to: T. Toyo'oka, Laboratory of Analytical and Bio-Analytical Chemistry, School of Pharmaceutical Sciences, and Global COE Program, University of Shizuoka, 52-1 Yada, Suruga-ku, Shizuoka 422-8526, Japan. E-mail: toyooka@u-shizuoka-ken.ac.jp

Jun Zhe Min, Laboratory of Analytical and Bio-Analytical Chemistry, School of Pharmaceutical Sciences, and Global COE Program, University of Shizuoka, 52-1 Yada, Suruga-ku, Shizuoka 422-8526, Japan. E-mail: junzhe@u-shizuoka-ken.ac.jp

[a] Laboratory of Analytical and Bio-Analytical Chemistry, School of Pharmaceutical Sciences, and Global COE Program, University of Shizuoka, 52-1 Yada, Suruga-ku, Shizuoka 422-8526, Japan

[b] Division of Pharmacognosy, Phytochemistry and Narcotics, National Institute of Health Sciences, 1-18-1 Kamiyoga, Setagaya-ku, Tokyo 158-8501, Japan

Abbreviations used: HDV, hydrodynamic voltammaogram; MECD, multi-channel electrochemical detection.

(*Salvia divinorum*) were listed as a new category, 'Designated Substances' (Shitei-Yakubutsu) in April 2007. Eleven substances were also added to this category in 2008 and 2009. Therefore, a rapid, simple, sensitive and simultaneous determination method is required for the detection and trace analysis of these designated substances in real samples.

The major methods for the determination of illegal drugs are simple inspection kits utilizing the antigen–antibody reaction, but the types of illegal drugs that can be inspected in this way are limited to stimulants, opium and cannabis. A coloration reaction has also been adopted for the screening various illegal drugs in the USA and Japan (US National Institute of Justice 2009; Satou 2009). However, most of the kits and the coloration tests are limited to the inspection of only two or three kinds of drugs. Therefore, new detection methods which could confirm and distinguish drugs having similar chemical structures are urgently required. However, the number of simultaneous determination methods, which are simple and sensitive, for various tryptamine, phenethylamine and piperazine analogs is quite limited. HPLC-FL (Nakamura *et al.*, 2007; Tomita *et al.*, 2006), GC-MS (Awad *et al.*, 2005; Theobald and Maurer 2006; Theobald *et al.*, 2005; Lin *et al.*, 2003; Balikova, 2005; Kikura-Hanajiri *et al.*, 2005, 2007) and LC-MS (Leung *et al.*, 2007; Gottardo *et al.*, 2007; Nakashima 2005; Nishida *et al.*, 2006; Jimenez *et al.*, 2006; Carrera *et al.*, 2007; Appollonio *et al.*, 2006; Wu *et al.*, 2008) are the most convenient methods for distinguishing the drugs. In our previous reports, fluorescence labeling methods by LC separation and TOF-MS detection were developed for the designated substances (Min *et al.*, 2008, 2009). Furthermore, determinations of phenethylamine containing products sold in the past were successfully performed by these methods. However, these methods require the fluorescence labeling of the designated substances in a sample before separation and detection. In addition, a high-performance analytical system including MS is required for the determination.

Since the designated substances possess an allylether, arylamine and indole in the structures and seem to be active for electrochemical oxidation, the simultaneous determination of 31 kinds of substances, i.e. 11 tryptamines, 14 phenethylamines, two piperazines and four others, was tried by HPLC with multichannel electrochemical detection (MECD). Furthermore, the designated substances in several real products, sold in Japan before the regulation, were also determined as an application of the proposed methods.

Experimental

Materials and Reagents

The hydrochloric acid salts of 31 designated substances, i.e. *N*-[2-(5-methoxy-1*H*- indol-3-yl)-ethyl]-*N*-propylpropan-1-amine (5-MeO-DPT), *N*-[2-(5-methoxy-1*H*-indol-3-yl)ethyl]-*N*-methylpropan-2-amine (5-MeO-MIPT), 1-(5-methoxy-1*H*-indol-3-yl)propan-2-amine (5-MeO-AMT), *N*-[2-(5-methoxy-1*H*-indol-3-yl)-ethyl]-*N*-vinylethenamine (5-MeO-DALT), 2-(5-methoxy-1*H*-indol-3-yl)-*N*,*N*-dimethylethanamine (5-MeO-DMT), 3-[2-(diisopropylamino)ethyl]-1*H*-indol-4-yl acetate (4-AcO-DIPT), 3-[2-(diisopropylamino)ethyl]-1*H*-indol-4-ol (4-OH-DIPT), *N*-[2-(1*H*-indol-3-yl)ethyl]- *N*-propylpropan-1-amine (DPT), *N*-[2-(1*H*-indol-3-yl)ethyl]-*N*-isopropylpropan-2-amine (DIPT), *N*-ethyl-*N*-[2-(5-methoxy-1*H*-indol-3-yl)ethyl]propan-2-amine (5-MeO-EIPT), *N*-[2-(1*H*-indol-3-yl)ethyl]-*N*-methylpropan-2-amine (MIPT), 4-chloro-2,5-dimethoxyphene-thylamine (2C-C), 4-ethyl-2,5-dimethoxyphenethylamine (2C-E), 4-iodo-2,5-dimethoxyphenethylamine (2C-I), 4-ethylthio-2,5-dimethoxyphenethylamine (2C-T-2), 4-isopropylthio-2,5-dimethoxyphenethylamine (2C-T-4), 1-(4-chloro-2,5-dimethoxyphenyl) propan-2-amine (DOC), 1-[4-(ethylthio)-2,5- dimethoxyphenyl]propan-2-amine (ALEPH-2), 1-(4-iodo-2,5-dimethoxyphenyl)propan-2-amine (DOI), *N*-methyl-1-*p*-tolypropan-2-amine (*N*-Me-FMP), 3,4-methylenedioxymethamphetamine (MMDA-2), *N*-methyl-1-(3,4-methylenedioxyphenyl)butan-3-amine (HMDMA), 2,4,6-trimethoxyamphetamine (TMA-6), 1-(3,4-methylenedioxyphenyl) butan-2-amine (BDB), 4-methoxymethamphetarnine (PMMA), 1-(4-methoxyphenyl)piperazine (4-MPP), 1-benzyl-4-methylpiperazine (MBZP), 1-(benzo[d][1,3]dioxol-5-yl)2-(pyrrolidin-1-yl)pentan-1-one (MDPV), 1-(benzo[d][1,3] dioxol-5-yl)-2-(ethylamino)propan-1-one (Bk-MDEA), 2-(ethylamino) -1-phenylpropan-1-one (*N*-ethylcathinone) and 2,3-dihydro-1*H*-inden-2-amine (indan-2-amine) were obtained from the National Institutes of Health Sciences (Tokyo, Japan; Fig. 1). Six real samples (Products 1–6), sold as legal substances on the Japanese market in 2006, were used for the determination of the designated substances as a real application. Methanol (CH_3OH), acetonitrile (CH_3CN) and tetrahydrofuran (THF) were of special reagent grade (Kanto Chemicals, Tokyo, Japan). Potassium phosphate dibasic (K_2HPO_4), potassium phosphate monobasic (KH_2PO_4) and phosphoric acid (H_3PO_4) were obtained from Wako Pure Chemical (Osaka, Japan). All other chemicals were of analytical-reagent grade and used without further purification. De-ionized and distilled water was used throughout the study (Aquarius pwu-200 automatic water distillation apparatus; Advantec, Tokyo, Japan).

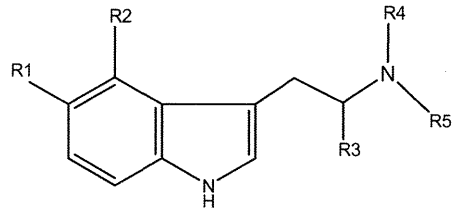
HPLC-MECD System

A Shimadzu (Kyoto, Japan) HPLC system consisting of two LC-20AD pumps, an auto injector ($SIL\text{-}20AC_{HT}$) and a degasser ($DGU\text{-}20A_3$) was used. Reversed-phase columns, i.e. TSK-gel ODS-100V (250 × 4.6 mm, i.d., 3 μm; Tosoh, Tokyo, Japan), and Cadenza CD-C18 (250 × 4.6 mm i.d., 3 μm; Imtakt Co. Ltd, Kyoto, Japan), were chosen for the separation optimization of the designated substances. A coulometric electrode array detector (Model 5600A CoulArray, ESA Inc., Chelmsford, MA, USA) equipped with 16 channel cell electrodes (model 6210, porous graphite working electrode) was used for the detection. The column and 16 electrodes were heated at 40°C. The collected data were processed with Windows 32 software (version 3.01) for CoulArray. An HM-50G pH meter (DKK-TOA Co., Tokyo, Japan) was used for pH adjustment of the buffer solution.

Simultaneous Separation of Designated Substances

Total separation of designated substances by single chromatographic run. The stock solutions of 31 designated substances were diluted with water to a 300 μg/mL concentration. An equal volume of each solution was mixed and an aliquot (10 μL) was injected into HPLC-MECD system. The mobile phases A and B consisted of 31.4 mM potassium phosphate buffer–CH_3OH–CH_3CN (95:4:1; pH 6.7) and 60 mM potassium phosphate buffer–CH_3OH–CH_3CN (50:40:10; pH 6.7), respectively. The separation was performed using a reversed-phase ODS column (TSK-gel ODS-100V, 250 × 4.6 mm, i.d., 3 μm) with gradient elution of 25% B (0–20 min), 10% B (20–60 min) and 10–70% B (60–240 min) at the flow rate of 1.0 mL/min. The applied potentials of the 16 channel electrodes were set at 0, 90, 180, 270, 360, 450, 540, 630, 720, 810, 900, 990, 1080, 1170, 1260 and 1350 mV (Table 1).

Group separations of designated substances. The stock solutions of the tryptamine analogs (group 1: 5-MeO-DPT, 5-MeO-MIPT, 5-MeO-AMT, 5-MeO-DALT, 5-MeO-DMT, 4-AcO-DIPT, 4-OH-DIPT, DPT, DIPT, 5-MeO-EIPT and MIPT), phenethylamine analogs (group 2: 2C-C, 2C-E, 2C-I, 2C-T-2, 2C-T-4, DOC, ALEPH-2, DOI, *N*-Me-FMP, MMDA-2, HMDMA, TMA-6, BDB and PMMA), and piperazine analogs and other substances (group 3: 4-MPP, MBZP, MDPV, Bk-MDEA, *N*-ethylcathinone and indan-2-amine) were diluted with water to a 300 μg/mL concentration. An equal volume of each solution in groups 1–3 was mixed and an aliquot (10 μL) was injected into HPLC-MECD system. The HPLC and the MECD conditions are listed in Table 1.



Tryptamines

No.	Substance	R1	R2	R3	R4	R5
1	MIPT	H	H	H	CH_3	$CH(CH_3)_2$
2	DPT	H	H	H	$CH_2CH_2CH_3$	$CH_2CH_2CH_3$
3	DIPT	H	H	H	$CH(CH_3)_2$	$CH(CH_3)_2$
4	5-MeO-AMT	OCH_3	H	CH_3	H	H
5	5-MeO-DMT	OCH_3	H	H	CH_3	CH_3
6	5-MeO-MIPT	OCH_3	H	H	CH_3	$CH(CH_3)_2$
7	5-MeO-DPT	OCH_3	H	H	$CH_2CH_2CH_3$	$CH_2CH_2CH_3$
8	5-MeO-DALT	OCH_3	H	H	$CH_2CH{=}CH_2$	$CH_2CH{=}CH_2$
9	4-OH-DIPT	H	OH	H	$CH(CH_3)_2$	$CH(CH_3)_2$
10	4-AcO-DIPT	H	$OCOCH_3$	H	$CH(CH_3)_2$	$CH(CH_3)_2$
11	5-MeO-EIPT	OCH_3	H	H	CH_2CH_3	$CH(CH_3)_2$

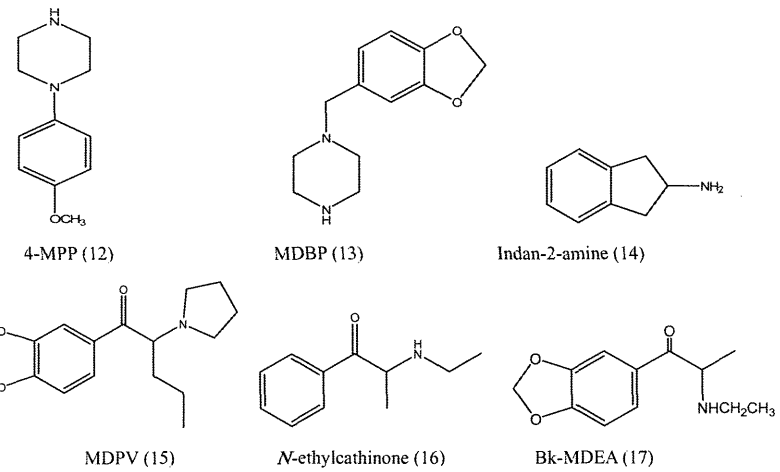


Figure 1. Structures of tested hallucinogenic designated substances (tryptamines, phenethylamines, piperazines and others).

Hydrodynamic Voltammogram

The stock solution of each designated substance was diluted with water to a 250 μg/mL concentration, and a 10 μL portion was then injected to HPLC-MECD system. The HPLC conditions were according to the separation conditions in each group.

Limit of Detection

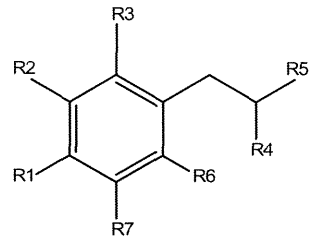
The limits of detection (LODs) of the designated substances at the recommended applied voltages were determined as a signal-to-noise ratio of 3 (S/N = 3). The stock solutions of the 31 designated substances were diluted with water to a series of concentrations (1.71 ng to 11.7 μg/mL). The LODs were calculated from a comparison of the noise level and the peak height on the suitable chromatograms which detected the trace amounts.

Calibration Curves

A series of working solutions of BDB (1.0–25 μg/mL), 2C-I (3.0–40 μg/mL), MMDA-2 (5.0–60 μg/mL), 5-MeO-AMT (3.0–200 μg/mL), 5-MeO-MIPT (0.1–2.0 μg/mL) and 4-OH-DIPT (1.0–10 μg/mL) were prepared by the dilution of the stock solution. Each solution was tested at five different concentrations. The peak heights based on the MECD response were plotted vs the absolute amounts of the substances. The RSDs (%) for each concentration were calculated from three determinations (n = 3).

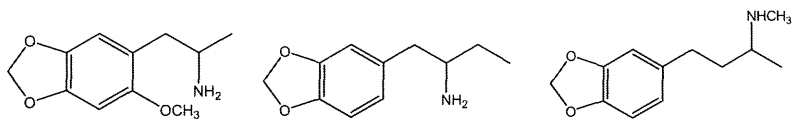
Accuracy and Precision by Intra-day and Inter-day Assay

The accuracy and precision of the intra-day and inter-day assays were tested using six designated substances. The accuracy (%) and precision (RSD%) were evaluated using three different concentrations in the ranges of 5–25 μg/mL (BDB), 3.0–40 μg/mL (2C-I), 10–60 μg/mL (MMDA-2), 5.0–20 μg/mL (5-MeO-AMT), 0.1–2.0 μg/mL (5-MeO-MIPT) and 2.0–10 μg/mL (4-OH-DIPT). The determinations were repeated three times within a day and between days. The accuracy (%) at each concentration was calculated from the calibration curves described in 'Calibration Curves'. The precision for each concentration was also calculated from the SD values for the three replicated determinations.



Phenethylamines

No.	Substance	R1	R2	R3	R4	R5	R6	R7
18	2C-I	I	OCH_3	H	H	NH_2	OCH_3	H
19	2C-C	Cl	OCH_3	H	H	NH_2	OCH_3	H
20	2C-E	CH_2CH_3	OCH_3	H	H	NH_2	OCH_3	H
21	2C-T-2	SCH_2CH_3	OCH_3	H	H	NH_2	OCH_3	H
22	2C-T-4	$SCH(CH_3)_2$	OCH_3	H	H	NH_2	OCH_3	H
23	TMA-6	OCH_3	H	OCH_3	CH_3	NH_2	OCH_3	H
24	DOI	I	OCH_3	H	NH_2	CH_3	OCH_3	H
25	PMMA	OCH_3	H	H	CH_3	$NHCH_3$	H	H
26	*N*-Me-FMP	F	H	H	$NHCH_3$	CH_3	H	H
27	DOC	Cl	OCH_3	H	NH_2	CH_3	OCH_3	H
28	ALEPH-2	SCH_2CH_3	H	OCH_3	CH_3	NH_2	H	OCH_3



MMDA-2 (29) BDB (30) HMDMA (31)

Figure 1. *Continued.*

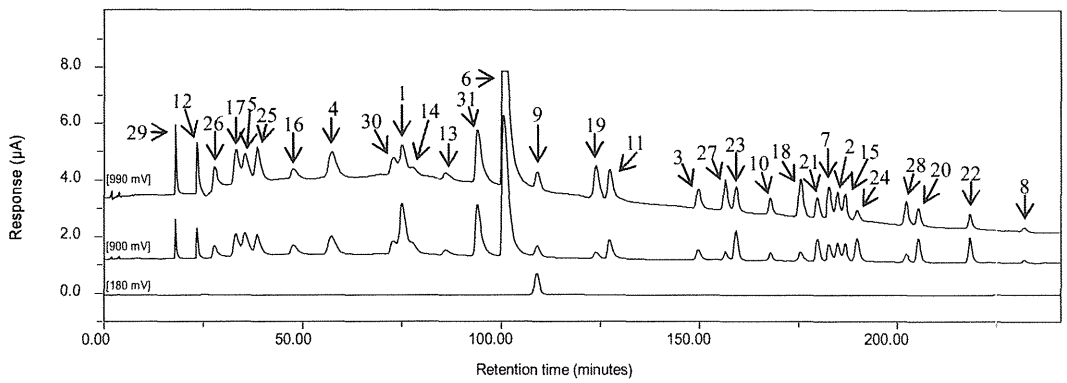


Figure 2. Chromatograms of 31 designated substances by HPLC-MECD 1: MIPT, 2: DPT, 3: DIPT, 4: 5-MeO-AMT, 5: 5-MeO-DMT, 6: 5-MeO-MIPT, 7: 5-MeO-DPT, 8: 5-MeO-DALT, 9: 4-OH-DIPT, 10: 4-AcO-DIPT, 11: 5-MeO-EIPT, 12: 4-MPP, 13: MDBP, 14: Indan-2-amine, 15: MDPV, 16: *N*-ethylcathinone, 17: Bk-MDEA, 18: 2C-I, 19: 2C-C, 20: 2C-E, 21: 2C-T-2, 22: 2-C-T-4, 23: TMA-6, 24: DOI, 25: PMMA, 26: *N*-Me-FMP, 27: DOC, 28: ALEPH-2, 29: MMDA-2, 30: BDB, 31: HMDMA. HPLC-MECD conditions are the same as those in Table 1(1).

Determination of Designated Substances in Real Samples

Products 1–6, sold in the past as legal substances in Japan, were used for the determination of the designated substances. One milligram each of the solid samples (products 1, 3, 4 and 5) was dissolved in 1.0 mL of a 50% methanol solution, sonicated for 10 min and then centrifuged at 2000 rpm for 10 min. After the centrifugation, the separated supernatant was filtered through a 0.45 μm membrane. The solutions were diluted 100 times with the initial solution of the mobile-phase and injected into the HPLC-MECD system. Because products 2 and 6 were liquid samples, the solution was first diluted 10 times with 50% methanol and then centrifuged at 2000 rpm for 10 min. After the centrifugation, the supernatant solution was filtered through a 0.45 μm membrane. The solution was diluted 10 times with initial solution of the

Table 1. Conditions of HPLC-MECD

(1) Total analysis	
Column	TSK-gel ODS-100V (3 μm, 250 × 4.6 mm, i.d., TOSOH)
Mobile phase A	31.4 mM potassium phosphate–CH_3OH–CH_3CN = 95:4:1 (pH 6.7)
Mobile phase B	60 mM potassium phosphate–CH_3OH–CH_3CN = 50:40:10 (pH 6.7)
Gradient elution	B. conc. 25% (0–20 min), 10% (20.1–60 min), 10–70% (60–240 min)
Column temperature	40°C
Flow rate	1.0 mL/min
Detector	Coularray
Electrode cell potential	0–1350 mV
Injection volume	10 μL
(2) Analysis for tryptamine analogues	
Mobile phase A	31.4 mM potassium phosphate–CH_3OH–CH_3CN = 95:4:1 (pH 3.5)
Mobile phase B	60 mM potassium phosphate–CH_3OH–CH_3CN = 50:40:10 (pH 3.5)
Gradient elution	B. conc. 20–60% (0–40 min), 60–90% (40–55 min)
Other HPLC-MECD conditions are the same as those in (1).	
(3) Analysis for phenethylamin analogues	
Mobile phase A	31.4 mM potassium phosphate–CH_3OH–CH_3CN = 95:4:1 (pH 3.5)
Mobile phase B	60 mM potassium phosphate–CH_3OH–CH_3CN = 50:40:10 (pH 3.5)
Gradient elution	B. conc. 65% (0–10 min), 80% (10–50 min)
Flow rate	0.5 mL/min
Other HPLC-MECD conditions are the same as those in (1).	
(4) Analysis for piperazine analogues and others	
Mobile phase A	31.4 mM potassium phosphate–CH_3OH–CH_3CN = 95:4:1 (pH 3.5)
Mobile phase B	60 mM potassium phosphate–CH_3OH–CH_3CN = 50:40:10 (pH 3.5)
Gradient elution	B. conc. 55% (0–10 min), 55–95% (10–15 min), 95% (15–20 min)
Flow rate	0.8 mL/min
Other HPLC-MECD conditions are the same as those in (1).	

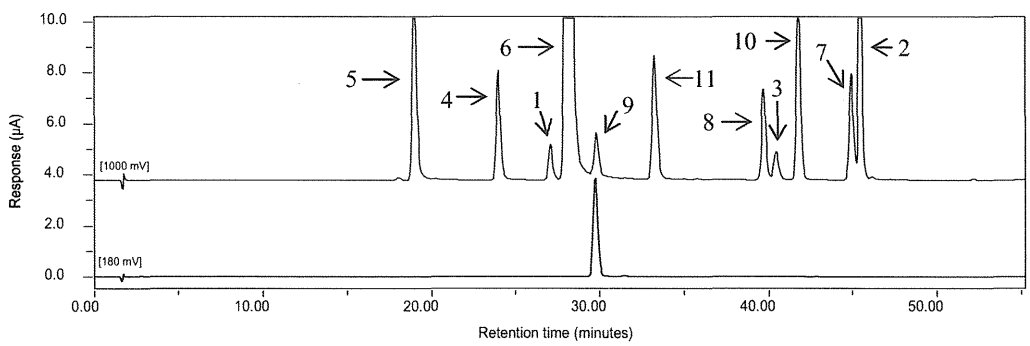


Figure 3. Chromatograms of tryptamines by HPLC-MECD 1: MIPT, 2: DPT, 3: DIPT, 4: 5-MeO-AMT, 5: 5-MeO-DMT, 6: 5-MeO-MIPT, 7: 5-MeO-DPT, 8: 5-MeO-DALT, 9: 4-OH-DIPT, 10: 4-AcO-DIPT, 11: 5-MeO-EIPT. HPLC-MECD conditions are the same as those in Table 1(2).

mobile phase and injected to HPLC-MECD system, as described above. The designated substances in the products were identified from the comparison of the retention times and the hydrodynamic voltammaogram (HDV) curves of authentic substances. The amounts of the designated substances in the products were also determined by the calibration curves.

Results and Discussion

Several screening methods, such as GC-MS and LC-MS, have been developed for the designated substances and successfully applied to real samples. Although these methods, which can confirm the molecular mass, are excellent for the discrimination of the designated substances, the analytical system including a MS instrument is rather expensive and cannot be used in many laboratories. The LC-UV method is thus adopted in such laboratories.

The designated substances tested in this study possess various functional groups, such as aliphatic and aromatic amines and allyl ether, in their structures (Fig. 1). In general, these functional groups are electrochemically active, although the response varies. Therefore, the designated substances, i.e. tryptamines, phenethylamines and piperazines, seem to be detectable by electrochemical oxidation. Based on this idea, the determination of these substances separated by a reversed-phase HPLC was attempted by online electrochemical oxidation. Since the ease of oxidation seems to be different in each substance, an MECD

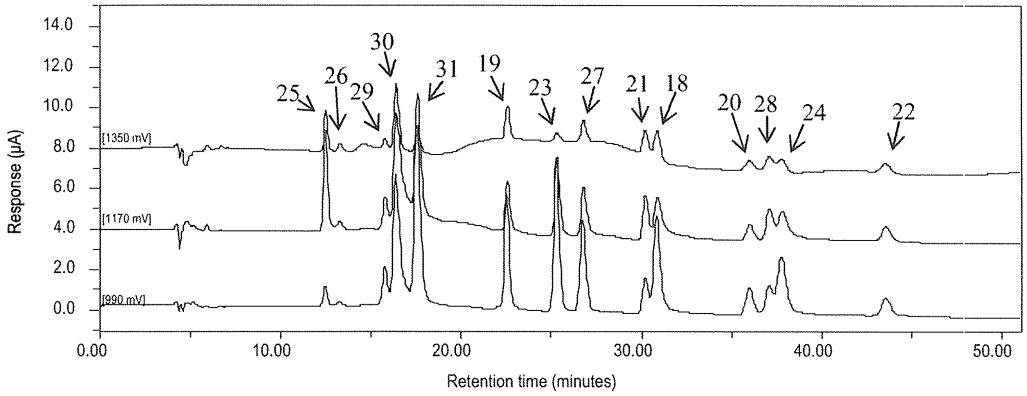


Figure 4. Chromatograms of phenethylamines by HPLC-MECD 18: 2C-I, 19: 2C-C, 20: 2C-E, 21: 2C-T-2, 22: 2C-T-4, 23: TMA-6, 24: DOI, 25: PMMA, 26: N-Me-FMP, 27: DOC, 28: ALEPH-2, 29: MMDA-2, 30: BDB, 31: HMDMA. HPLC-MECD conditions are the same as those in Table 1(3).

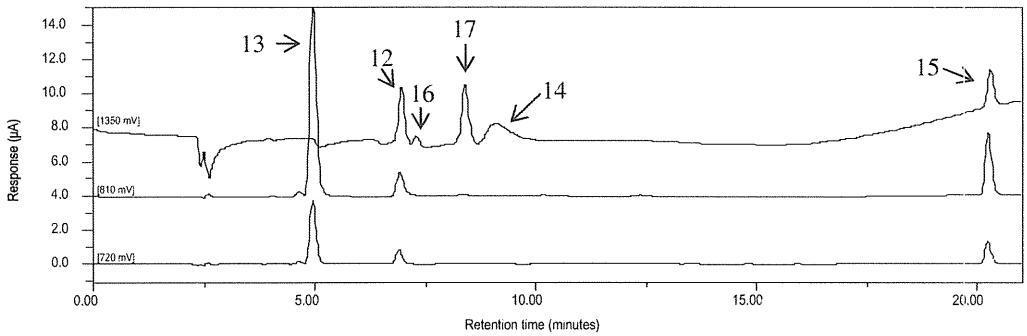


Figure 5. Chromatograms of piperazines and other designated substances by HPLC-MECD 12: 4-MPP, 13: MDBP, 14: Indan-2-amine, 15: MDPV, 16: *N*-ethylcathinone, 17: Bk-MDEA. HPLC-MECD conditions are the same as those in Table 1(4).

Table 2. Limit of detection of designated substances for the applied voltages

Designated substance	Detection voltage (mV)	LOD(pg)	Designated substance	Detection voltage (mV)	LOD (pg)
5-MeO-DPT	1000	93.8	DPT	1000	75.0
5-MeO-MIPT	1000	17.7	4-OH-DIPT	1000	81.5
5-MeO-AMT	1000	30.0	MIPT	1000	93.8
5-MeO-DALT	1000	28.6	4-AcO-DIPT	1000	37.5
5-MeO-DMT	1000	86.7	DIPT	1000	83.3
5-MeO-EIPT	1000	31.6			
2C-E	1200	8.40×10^3	2C-I	1200	1.60×10^3
2C-T-2	1200	990	DOI	1200	359
2C-T-4	1200	2.80×10^3	2C-C	1200	717
ALEPH-2	1200	1.15×10^3	DOC	1200	387
HMDMA	1200	300	*N*-Me-FMP	1200	18.2×10^3
BDB	1200	440	PMMA	1200	500
MMDA-2	1200	3.75×10^3	TMA-6	1200	221
MDPV	1100	250	*N*-Ethylcathinone	1100	1.47×10^3
4-MPP	1000	188	Indan-2-amine	1200	117×10^3
MDBP	1100	167	Bk-MDEA	1200	586

LOD, limit of detection (S/N = 3).

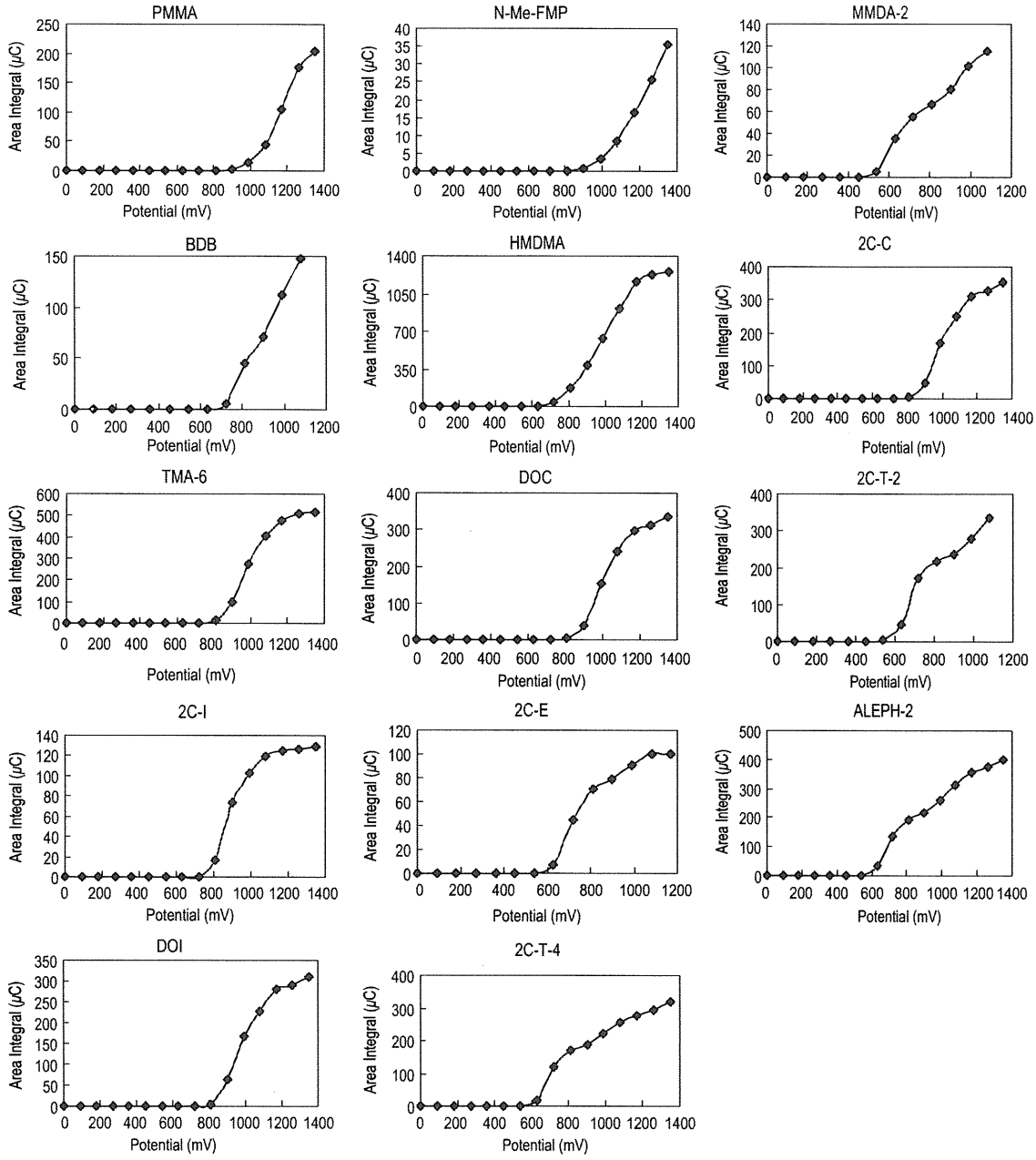


Figure 6. HDVs of phenethylamines obtained from HPLC-MECD HPLC-MECD conditions are the same as those in Table 1(3).

system was adopted for the determination of the substances eluted from the column.

Separation and Detection of 31 Designated Substances

The various conditions that affect the separation and detection of the designated substances were optimized using a few ODS columns, such as ODS-100V and Cadenza CD-C18. For the electrochemical determination, the existence of an electrolyte is essential for the efficient detection. Therefore, potassium phosphate buffer was added to the mobile phases in the present study. Various factors (e.g. buffer concentration, pH, modifier species, modifier concentration and gradient elution condition, etc.) affecting the separation were first tested. The separations were influenced by the concentrations of the buffer, modifier (methanol, acetonitrile and THF), gradient patterns, etc. The following conditions were finally selected for the simultaneous separation of all the designated substances: column, ODS-100V (4.6 × 250 mm, i.d., 3 μm); mobile phase A, 31.4 mM potassium phosphate buffer–CH_3OH–CH_3CN (95:4:1), pH 6.7; mobile phase B, 60 mM potassium phosphate buffer–CH_3OH–CH_3CN (50:40:10, pH 6.7). Although a simple gradient elution is recommended for such chromatographic separation, the peak-to-peak separation of several substances was insufficient. Therefore, the following linear gradient profile was selected for the separation of 31 designated substances: 25% B (0–20 min), 10% B (20–

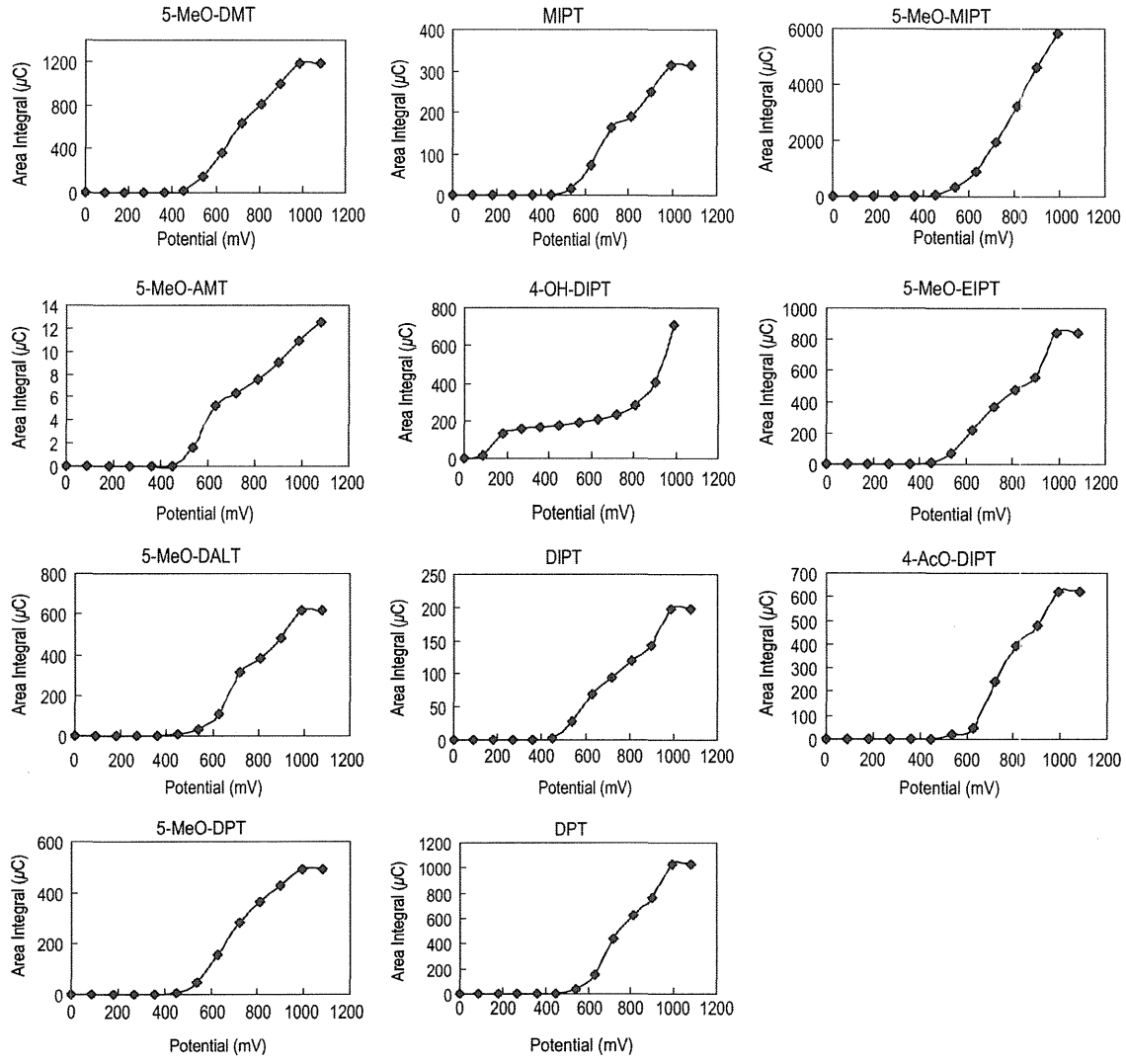


Figure 7. HDVs of tryptamines obtained from HPLC-MECD. HPLC-MECD conditions are the same as those in Table 1(2).

60 min) and 10–70% B (60–240 min) at the flow rate of 1.0 mL/min. Under these conditions, the 31 kinds of designed substances were well separated and detected (Fig. 2). However, the chromatographic run time was too long (240 min). Therefore, the designated substances were classified into three groups: (1) tryptamine analogs; (2) phenethylamine analogs; and (3) piperazine analogs and others. Then the designated substances in each group were separated using the optimal conditions.

The tryptamine analogs (group 1) were separated by the following gradient elutions (0–60% B at 0–40 min and 60–90% B at 40–55 min) using 31.4 mM potassium phosphate buffer–CH_3OH–CH_3CN (95:4:1; pH 3.5; mobile phase A) and 60mM potassium phosphate buffer–CH_3OH–CH_3CN (50:40:10; pH 3.5; mobile phase B). Figure 3 shows the chromatograms obtained from the 11 types of tryptamines.

The simultaneous separation of 14 kinds of phenethylamines (group 2) was also carried out using the same column and mobile phases. The optimized gradient elution conditions were 65% B (0–10 min) and 80% B (10–50 min). When the flow rate of the mobile phase was 1.0 mL/min, 2C-E, ALEPH-2 and DOI could not be separated well. The separation was improved by a low flow rate (0.5 mL/min). However, a long time was required for the separation due to the low flow rate. The chromatogram is shown in Fig. 4.

A good separation of the piperazine analogs and other substances was observed from the gradient elution of 55% B (0–10 min), 55–95% B (10–15 min) and 95% B (15–20 min) at the low flow rate of 0.8 mL/min. A typical chromatogram is shown in Fig. 5. The piperazines and the others were simultaneously separated within 25 min.

Determination of HDV and LOD

Judging from the chromatograms of Figs 2–5, the responses of the designated substances seemed to be fairly different in the applied potential range, as was expected. For example, 4-OH-DIPT was easily oxidized at a relatively low applied voltage (180 mV). Therefore, the HDVs of each substance were determined. As shown in Figs 6–8, the shapes of the HDV curves varied

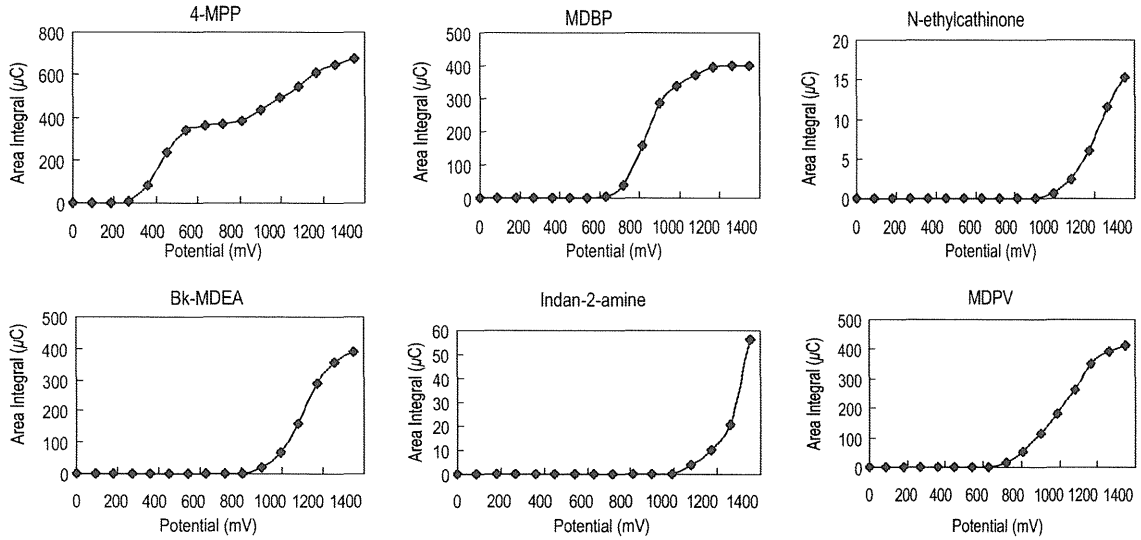


Figure 8. HDVs of piperazines and other designated substances obtained from HPLC-MECD. HPLC-MECD conditions are the same as those in Table 1(4).

for each designated substance. The exact reason for the variance is not obvious. However, the difference seems to be dependent on the functional groups such as indole and arylamine in the structures of the designated substances (Fig. 1). The results suggest that a rough estimation of the designated substances in real samples might be possible from the HDV pattern.

The LODs (S/N = 3) of the designated substances were determined at suitable applied voltages obtained from the optimization. The results of LOD at suitable voltages are shown in Table 2. Although the sensitivities of all the substances seemed to be higher than those of the UV detection, a wide variation in the LODs from 17.1 pg (5-MeO-MIPT) to 117 ng (indan-2-amine) was observed. The tryptamine analogs tended to be sensitive, but the LODs of the phenethylamines were strongly dependent on each substance.

Application to the Real Samples Containing Designated Substances

The determination of designated substances in several real samples was carried out as an application of the present methods. The designated substances in liquid samples, i.e. products 2 (colorless liquid) and 6 (yellow liquid), were simply diluted, filtered and determined by HPLC-MECD. In the case of the solid samples, i.e. products 1 (yellow powder), 3 (mushroom-like), 4 (orange powder) and 5 (brown solid), the designated substances were extracted with 50% CH_3OH, sonicated, centrifuged, filtered and then similarly determined by HPLC-MECD. Each solution was first subjected to HPLC-MECD system for total analysis. The kind of designated substances in these products were speculated from the retention times of the chromatogram obtained from the mixture of 31 authentic substances. Since the chromatographic run time in the total separation system is long, the assay of the designated substances in the products was carried out by group separations. The typical chromatograms obtained from these samples are shown in Figs 9 and 10. Phenethylamine analogs, i.e. BDB, MMDA-2 and 2C-I, were identified from products 1, 2 and 3, respectively (Fig. 9). On the other hand, tryptamine analogs, i.e. 5-MeO-AMT (product 4), 5-MeO-MIPT and 4-OH-DIPT (product 5) and 5-MeO-MIPT (product 6), were also detected (Fig. 10). In addition, unknown peaks were also detected in products 2 and 5. Judging from the retention times and HDV curves, the unknown peaks in Fig. 9(c) (product 2) and Fig. 10(c) (product 5) seemed to be not the designated substances targeted in this study.

The calibration curves were determined at five different concentrations (n = 3) involving the amounts speculated in the sample solutions. A good calibration curve was obtained from each designated substance by the group determination methods (Table 3). The accuracy (%) and precision (RSD%) by the group determination methods were also determined. The results of the intra-day and inter-day determinations at three different concentrations are shown in Table 4. A good accuracy (%) was obtained from all the designated substance. Although the RSDs (%) for the intra-day and inter-day assay were different for each designated substance, the values were less than 6.7%. The recovery test was not performed in the present study because there was information about the matrix in the products. The designated substances spiked in water were quantitatively recovered as shown in Table 4.

Based on the calibration curves, the concentrations of each designated substance were calculated as 0.77 mg/mg (BDB in product 1), 3.66 mg/mL (MMDA-2 in product 2), 0.018 mg/mg (2C-I in product 3), 13 μg/mg (5-MeO-AMT in product 4), 0.21 and 7.8 μg/mg (5-MeO-MIPT and 4-OH-DIPT in product 5) and 500 μg/mL (5-MeO-MIPT in product 6).

Although only the validation data of the designated substances which were detected in the real samples are shown in Tables 3 and 4, similar good accuracy, precision and calibration curves were obtained from the other designated substances. Therefore, the proposed methods seem to be applicable not only for the qualitative but also for quantitative determinations of unknown designated substance in real samples.

Conclusion

New screening methods based on online electrochemical oxidation have been developed for the designated substances. To the

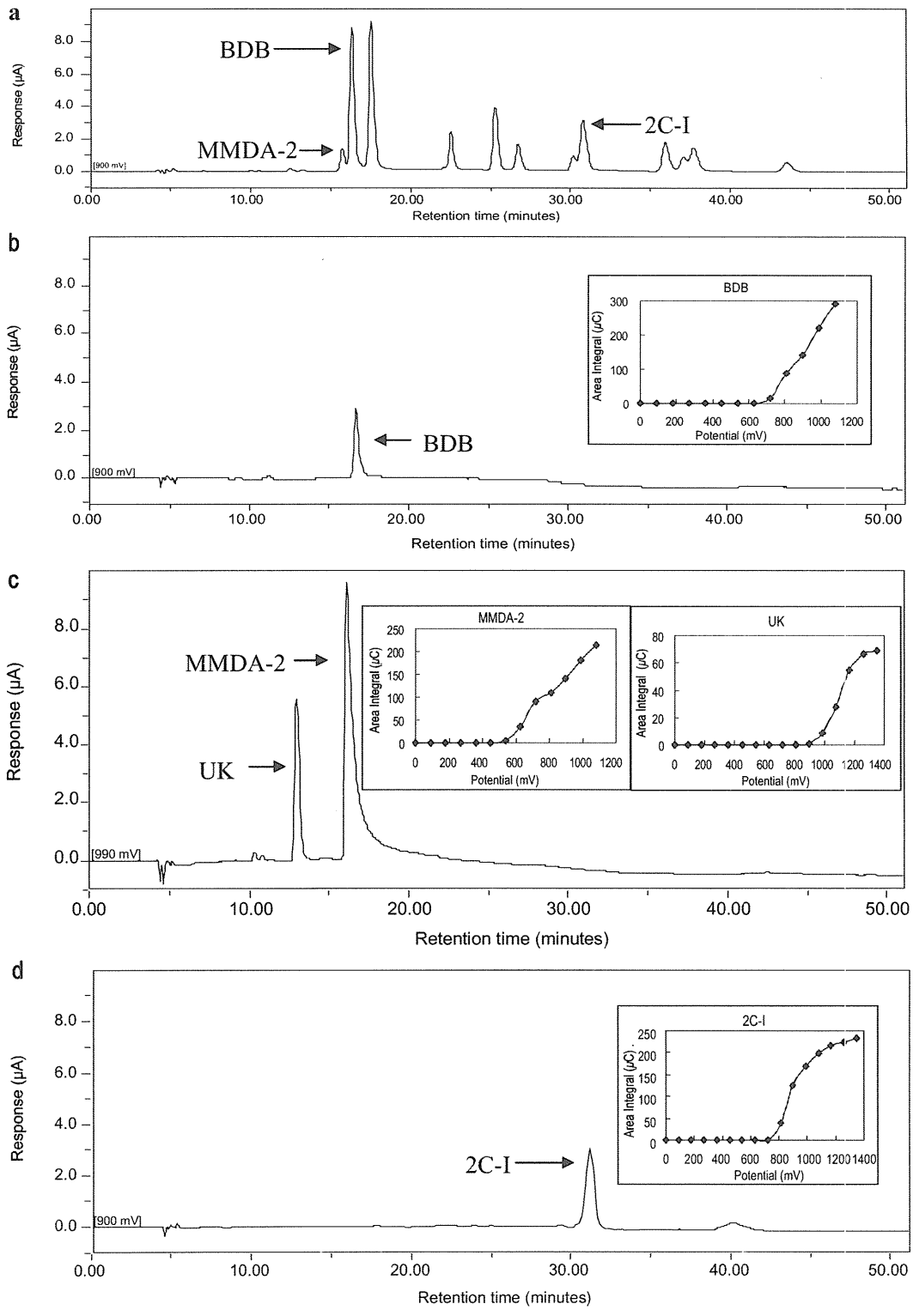


Figure 9. Chromatograms and HDVs obtained from real products containing phenethylamines by HPLC-MECD. (a) Standard phenethylamines; (b) Product 1 (yellow powder); (c) Product 2 (colorless liquid); (d) Product 3 (mashroom like). HPLC-MECD conditions are the same as those in Table 1(3).

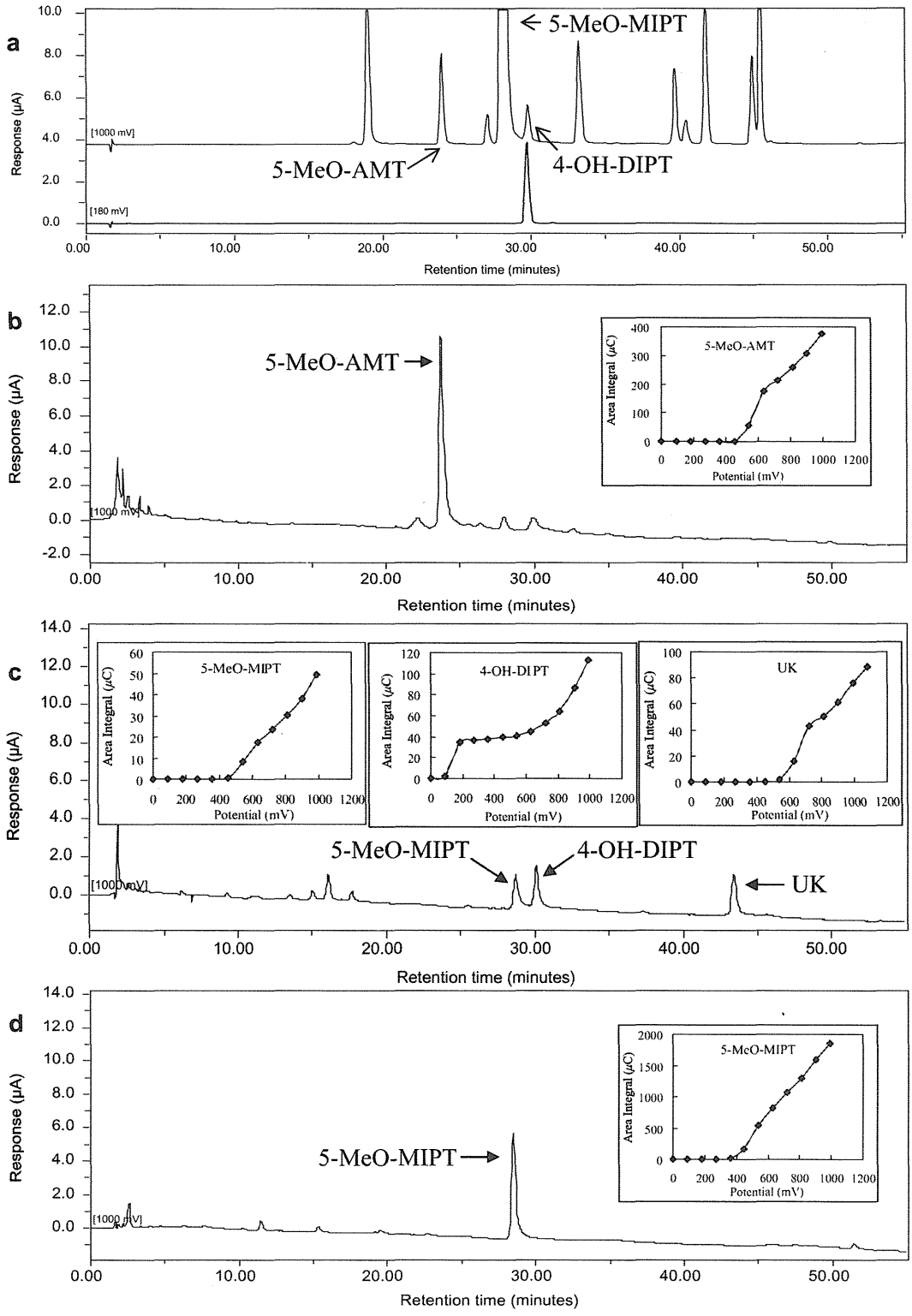


Figure 10. Chromatograms and HDVs obtained from real products containing tryptamines by HPLC-MECD. (a) Standard tryptamines; (b) Product 4 (orange powder); (c) Product 5 (brown solid); (d) Product 6 (yellow liquid). HPLC-MECD conditions are the same as those in Table 1(2).

Table 3 Calibration curves for designated substances by the group determination methods ($n = 3$)

Designated substance	Calibration range (ng)	Linear equation	Linearity (R^2)
BDB	10–250	$y = 0.7108x - 0.3276$	0.999
2C-I	30–400	$y = 0.3095x - 2.988$	0.999
MMDA-2	50–600	$y = 0.3394x - 5.1131$	0.999
5-MeO-AMT	30–200	$y = 1.8258x - 7.4046$	0.999
5-MeO-MIPT	1–20	$y = 20.15x + 0.092$	0.999
4-OH-DIPT	10–100	$y = 0.8391x - 2.1655$	1.000

Table 4. Accuracy and precision of intra-day and inter-day assays by the group determination methods

Designated substance	Amount (ng)	Intra-day assay ($n = 3$)			Inter-day assay ($n = 3$)		
		Mean ± SD	Accuracy %	RSD %	Mean ± SD	Accuracy %	RSD%
BDB	50	50.4 ± 0.19	100.8	0.38	50.5 ± 0.083	101.0	0.17
	100	102 ± 0.50	102.0	0.50	101 ± 0.22	101.0	0.22
	250	249 ± 0.10	99.60	0.077	249 ± 4.4	99.60	1.8
2C-I	30	28.7 ± 1.3	95.70	4.5	28.1 ± 1.2	93.70	4.1
	200	197 ± 2.4	98.50	1.2	198 ± 0.83	99.00	0.42
	400	402 ± 1.3	100.5	0.31	402 ± 1.8	100.5	0.45
MMDA-2	100	96.9 ± 2.3	96.90	2.3	99.4 ± 0.85	99.40	0.86
	300	302 ± 3.8	100.7	1.3	299 ± 4.7	99.70	1.6
	600	599 ± 1.7	99.80	0.28	601 ± 2.9	100.2	0.49
5-MeO-AMT	50	50.7 ± 0.058	101.4	0.12	50.3 ± 0.57	100.6	1.1
	100	99.8 ± 0.78	99.80	0.78	99.6 ± 0.96	99.60	0.96
	200	200 ± 0.29	100.0	0.15	200 ± 0.34	100.0	0.17
5-MeO-MIPT	1	0.930 ± 0.013	93.00	1.3	0.980 ± 0.067	98.00	6.7
	5	5.10 ± 0.057	102.0	1.1	5.08 ± 0.027	101.6	0.54
	10	10.0 ± 0.12	100.0	1.2	9.91 ± 0.090	99.10	0.91
	20	20.0 ± 0.050	100.0	0.25	20.0 ± 0.049	100.0	0.25
4-OH-DIPT	20	20.0 ± 0.19	100.0	0.95	20.2 ± 0.19	101.0	0.96
	50	50.1 ± 0.31	100.2	0.62	49.6 ± 0.61	99.20	1.2
	100	100 ± 0.32	100.0	0.32	100 ± 0.25	100.0	0.25

SD: standard deviation; RSD: relative standard deviation.

best of our knowledge, this is the first report of such methods for the simultaneous determination of various designated substances by online multi-channel electrochemical oxidation. The 31 designated substances, controlled as 'Shitei-Yakubutsu' by the Pharmaceutical Affairs Law in Japan, were simultaneously separated and detected within 240 min by reversed-phase chromatography by MECD. Furthermore, the designated substances, divided into three groups, (1) tryptamines, (2) phenethylamines and (3) others containing piperazines, were also successfully determined by HPLC-MECD. The separation and detection of the designated substances in groups 1 (11 tryptamines), 2 (14 phenethylamines) and 3 (6 substances containing piperazines) were totally performed within 50, 45 and 20 min, respectively. Therefore, 31 kinds of designated substances were determined using total separation and/or group separations. The methods were successfully used to determine the designated substances in six real samples. The proposed methods are based upon direct analysis without complicated pretreatments such as fluorescence labeling. The LOD (17.1 pg to 117 ng) of the proposed methods was dependent on the structure of each designated substance and not equally good sensitive. However, the determination of real samples seems to be possible, as demonstrated in the analyses of several products. Furthermore, the HPLC-MECD system may be useful for the rough identification of the designated substances in some real samples, because the HDV curve pattern is different for each substance. Consequently, we believe that the present methods are useful for the qualitative and quantitative analyses of various designated substances in real samples.

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