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# Development of a sensitive and quantitative analytical method for 1*H*-4-substituted imidazole histamine $H_3$-receptor antagonists utilizing high-performance liquid chromatography and dabsyl derivatization

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**Abstract**

A sensitive and versatile analytical method utilizing high-performance liquid chromatography (HPLC) and precolumn derivatization of 1*H*-4-substituted imidazole compounds is described. A HPLC method using 4-dimethylaminoazobenzene-4′-sulfonyl chloride (dabsyl chloride) and ultraviolet (UV) detection was developed for the analysis of histamine (HA) $H_3$-selective compounds in human plasma, rat plasma, or homogenized rat cortical tissue. The average intra- and inter-assay variability, over a range of 10 to 0.01 μg/ml, was determined to be acceptable. The lower limit of detection for the dabsylated ligands was estimated to be <1.0 ng/ml while the lower limit of quantitation (LLOQ) was determined to be 10 ng/ml of conjugate. This assay has demonstrated it's suitability for the sensitive quantitation of several structurally diverse 1*H*-4-substituted imidazole HA $H_3$-receptor antagonists in biological matrices for pharmacokinetic and biodistribution studies. 

*Keywords:* Derivatization, LC; Histamine $H_3$-receptor antagonists; Dabsyl chloride

## 1. Introduction

It has been suggested, since the elucidation of the $H_3$-receptor subtype [1,2] in the central nervous system (CNS), that the histamine (HA) $H_3$-receptor could provide a therapeutic target for a variety of central nervous system disorders including: antinociception, anxiety, epilepsy, satiety, arousal and vigilance [3–8]. In addition to central nervous system utility, HA $H_3$ receptors may have a functional role in the peripheral nervous system as well [9–12]. Functional studies of the HA $H_3$-receptor subtype have demonstrated the role of the receptor as a heteroreceptor on nonhistaminergic neurons for other vital biogenic amines and peptides that are known behavioral modifiers [13–18]. It is evident that investigation of the HA $H_3$ receptor, and ligands which modulate it, can provide novel avenues of treatment of a variety of central and peripheral nervous system pathologies.

Structure activity relationship studies of HA $H_3$-

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selective compounds have revealed that an imidazole head-group, a spacer containing various functional groups, and a hydrophobic tail-group are significant in $H_3$-receptor binding [19,20]. As a result, several new 1*H*-4-substituted imidazole $H_3$-selective compounds, with diverse structural configurations, were synthesized (Fig. 1). Quantification of these new compounds proved formidable for pharmacokinetic and biodistribution studies due to weak chromophores that do not permit UV detection to the low nanomole level.

Derivatization schemes for analysis of early $H_3$-receptor ligands such as thioperamide, using benzene [21], and (*R*)-α-methylhistamine, using σ-phthaladehyde [22], have been described. Although these assays are useful in quantifying thioperamide and (*R*)-α-methylhistamine, they are problematic in that benzene and σ-phthaladehyde conjugates are stable for only a very short time. Moreover, these assays utilize highly specific and selective HPLC conditions and equipment that do not lend themselves to the versatility needed to accurately quantify several structurally diverse compounds.

Dabsyl chloride is a well established UV-labeling agent that has been primarily used for the covalent bonding to, and quantitation of, amino acids [23–28] and peptides [29,30] but, has also shown utility in the quantitation of thiols, phenols, aliphatic hydroxyls, and imidazoles. Several methods using dabsyl chloride have been developed for the quantitation of physiological imidazole-containing compounds such as histamine [26], histidine [31,32], imidazole dipeptides [31], methylhistidine [31], and urocanic acid [32], although dabsyl chloride has never been used to quantify HA $H_3$-receptor selective compounds.

Therefore, the aim of the present study was to develop a selective and quantitative analytical method for 1*H*-4-substituted imidazole compounds that would incorporate a selective solid-phase extraction method with a simple precolumn derivatization scheme in conjunction with a reliable reversed-phase HPLC system. Subsequently, a versatile reversed-phase HPLC method, amenable for various 1*H*-4-substituted imidazole compounds and using dabsyl chloride as a chromophoric labeling agent, was developed (Fig. 2). Further, we have been able to correlate derivatization conditions of new 1*H*-4-sub-

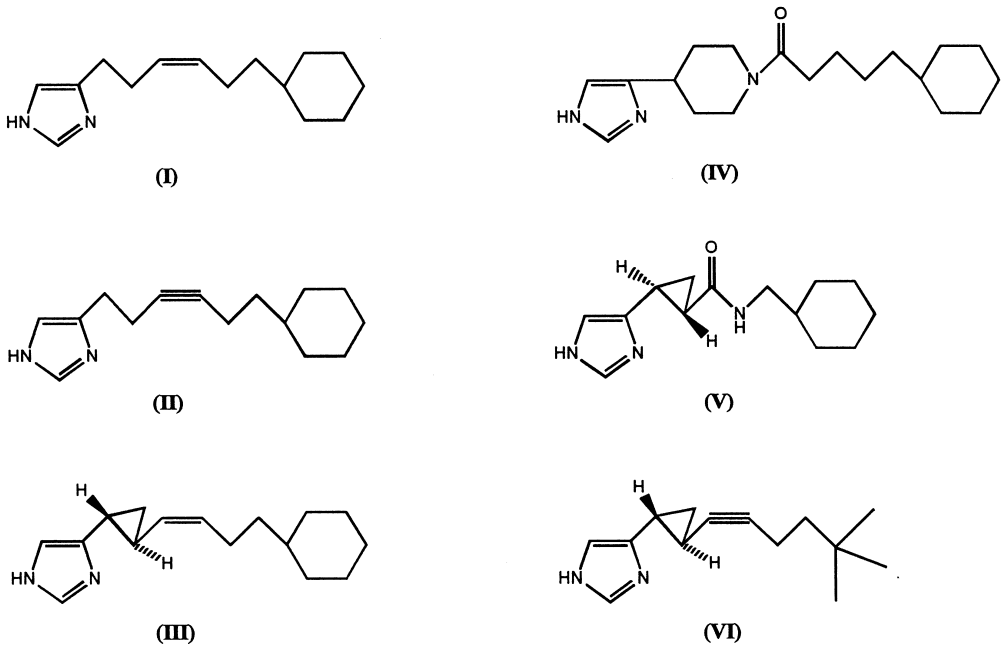


Fig. 1. Chemical structures of HA $H_3$-receptor selective compounds (I) GT-2227, (II) GT-2260, (III) GT-2209, (IV) GT-2016, (V) GT-2212, and (VI) GT-2331.

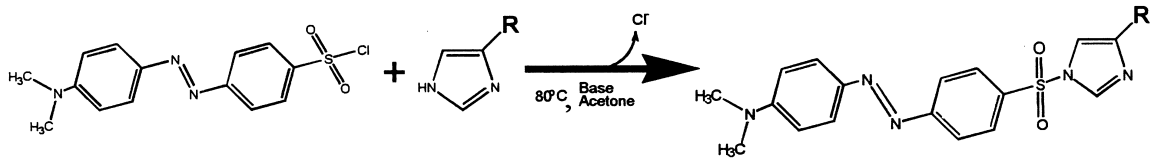


Fig. 2. Schematic representation of the *N*-1 substitution of 4-dimethylaminoazo benzene-4′-sulfonyl chloride to the imidazole head group of a 1*H*-4-substituted imidazole HA $H_3$-selective compound.

stituted imidazole compounds with theoretically derived single-point steric energy values from their respective energy-minimized stable conformations. Using these correlations, it is possible to estimate derivatization conditions of future 1*H*-4-substituted imidazole compounds and to determine the feasibility of mono-substituted or di-substituted compounds. Additionally, we have utilized theoretically derived log *P* values and experimentally derived retention times to give us the capability to estimate retention times of new compounds and multiple substituted compounds. Also, we have demonstrated the utility and improvement of hydrophilic–lipophilic-balanced solid-phase extraction of compounds over liquid–liquid and conventional $C_{18}$ extraction methods.

This assay provides a method that is validated, obtains picomolar sensitivity, and is versatile enough for quantitation of a broad range of 1*H*-4-substituted imidazole compounds. In the present study we have examined six structurally diverse 1*H*-4-substituted imidazoyl compounds that are selective $H_3$ antagonists. These compounds are currently undergoing safety testing for clinical trials after demonstrating favorable pharmacological profiles in preclinical testing. Presently, this assay will be utilized to elucidate the concentration of 1*H*-4-substituted imidazole $H_3$-selective drugs in biological samples for use in pharmacokinetic and biodistribution studies.

## 2. Experimental

### *2.1. Chemicals and reagents*

Compound I (*cis*-1-[1*H*-imidazol-4-yl]-6-cyclohexyl-hex-3-ene), compound II (4-(6-cyclohexylhex-3-ynyl)imidazole), compound III ((1*R*,2*R*)-*trans*-(1*H*-5-imidazoyl)cyclopropyl-6-cyclohexyl-3-hexene), compound IV (4-(1-cyclohexylpentanoyl-4-piperidyl), compound V ((1*R*,2*S*)-*trans*-*N*-(2-cyclohexylethyl)(2-imidazol-4-ylcyclopropyl)formamide), and compound VI ((1*R*,2*R*)-4-(2-(5,5-dimethylhex-1-ynyl)cyclopropyl)imidazole) were synthesized in our laboratory. 4-Dimethylaminoazobenzene-4′-sulphonyl chloride was purchased from Regis Technologies, (Morton Grove, IL, USA). Sodium acetate trihydrate was purchased from JT Baker (Phillipsburg, NJ, USA). All other reagents and chemicals were of chromatography grade and were purchased from and EM Science (Gibbstown, NJ, USA).

### *2.2. Instrumentation*

The HPLC system consisted of a LC 200 series quaternary pump, a 200 series column heater, a 200 series autosampler (Perkin Elmer, Norwalk, CT, USA) and a Dynamax UV-C variable UV–VIS detector (Rainin Instruments, Woburn, MA, USA). Data acquisition was carried out by a DECpc 433dxLP (Digital, Houston, TX, USA) computer station. While data integration was carried out by Perkin Elmer Turbochrom (v 4.1) software.

### *2.3. Chromatographic conditions*

Analysis was performed by utilizing a Prodigy (Phenomenex, Torrance, CA, USA) 5-μm ODS(2) guard column (30×3.2 mm I.D.) in series with a Prodigy 5-μm ODS(2) analytical column (150×3.2 mm I.D.). The column temperature was maintained at 40°C. Spectrophotometric detection was carried out at 479 nm. The mobile phase consisted of a three component system. Solvent A consisted of a 6:1 (v/v) 30 m*M* sodium acetate buffer: ACN solution (adjusted to pH 6.7 with 1 *M* acetic acid), solvent B consisted of isopropyl alcohol, while solvent C consisted of a ACN–TFA (0.01%) solution. All

mobile phase components were filtered through a 0.45-μm filter and sparged with helium prior to and through all analytical runs. Mobile phase flow was maintained at 0.4 ml/min. The gradient composition is shown in Table 1.

### 2.4. Hydrophobicity determinations of compounds

Relative hydrophobicity, expressed as the $\log_{10}$ of the water/octanol partition coefficient ($\log P$), of the parent compounds and their dabsylated derivatives were estimated with an additive-constitutive algorithm using $\log P$ software (Advanced Chemistry Development, Toronto, Canada). The algorithm is based on the well-characterized $\log P$ contributions of separate atoms, structural fragments, and intramolecular interactions between different fragments. Calculated $\log P$ values were compared to experimentally established HPLC retention times and used to estimate possible retention times of future compounds and their dabsylated conjugates.

### 2.5. Extraction procedure

Rat whole blood and tissue samples were collected in EDTA-treated vacutainers from euthanized adult male Sprague-Dawley rats all weighing approximately 300 g. Whole blood was immediately centrifuged for 15 min at 1900*g* on a Sorvall RT6000B refrigerated centrifuge (Du Pont Medical Products, Chicago, IL, USA) and separated plasma was transferred to polypropylene microfuge tubes and stored at −80°C until use. Concurrently, brain tissue was removed from the cranial cavity and hemisected with the subsequent excision and weighing of the cortical region. Each of the cortices was transferred to a polypropylene centrifuge tube and diluted to 30 mg/ml with phosphate buffered saline and homogenized with an ultrasonic tissue processor. The solution was then stored at −80°C until use.

Human whole blood samples were collected from a healthy male volunteer in EDTA-treated vacutainers. Blood was centrifuged for 15 min at 1900*g* on a Sorvall RT6000B refrigerated centrifuged. Subsequently, plasma was transferred to polypropylene tubes and stored at −80°C until use.

Plasma and brain homogenate samples were thawed and spiked with specified concentrations of 1*H*-4-substituted imidazole HA $H_3$-selective compounds for tests. Spiked samples were vortexed for 2 min and then allowed to stand for 20 min at 25°C. For compound extraction, 500 μl of the sample and 200 μl (10 μg/ml in water) of compound II, internal standard, was added to a 30 mg Oasis hydrophilic–lipophilic-balanced extraction cartridge (Waters, Milford, MA, USA) preconditioned with 1 ml of methanol and 1 ml of deionized water. Samples were drawn through the cartridge via a vacuum manifold using 20 kPa of pressure. Extraction cartridges were washed with 1 ml of a 5% methanol solution. Elution of compounds was accomplished by a 1-ml wash with a 100% acetone solution.

### 2.6. Steric energy determinations and derivatization conditions of dabsyl conjugates

Prior to derivatization, compounds were modeled three-dimensionally with and without dabsyl chloride covalently attached to all primary and secondary amines in an effort to determine optimized derivatization conditions and the feasibility of mono- and di-substituted dabsyl derivatives. A Pentium pro-

Table 1
HPLC gradient composition

| Time (min) | Solvent A (%)[a] | Solvent B (%)[a] | Solvent C (%)[a] | Curve |
|---|---|---|---|---|
| 0 | 60 | 32 | 8 | 0 |
| 10 | 40 | 48 | 12 | 1 |
| 25[b] | 40 | 48 | 12 | 0 |
| 5 | 60 | 32 | 8 | 1 |
| 10 | 60 | 32 | 8 | 0 |

[a] Solvent A consisting of a 6:1 (v/v) of 30 m*M* sodium acetate buffer and ACN, and Solvent B consisting of isopropyl alcohol, and Solvent C consisting of ACN, and 0.01% TFA.
[b] Routine analytical runs were accomplished by decreasing step two to 10 min resulting in a total run-time of 35 min.

cessor-driven computer system in conjunction with Chem3D Pro molecular modeling software v 3.5.1 (Cambridgesoft, Cambridge, MA, USA) were used to model the parent compounds and their respective dabsyl chloride conjugates. Using a semi-empirical method to calculate the quantum mechanical approximations, the energy-minimized stable conformations of the parent compounds and their derivatized conjugates were determined.

Using the energy-minimized stable conformations, the single-point steric energy value of the parent compound and derivatized conjugate was calculated. Differences in steric energy contributions between derivatized compounds and parent compounds and between cyclopropane-containing analogs and straight chain analogs were determined. These differences can be related to the amount of energy, as a function of time and heat, necessary to overcome the steric energy created by a di- or mono-substituted dabsyl derivative. Moreover, from these values estimation of time and heat needed for the coupling of structurally diverse 1*H*-4-substituted imidazole compounds to dabsyl chloride can be made.

The use of steric energy calculations in the present assay, was used to assist in the determination of derivatization conditions, specifically incubation time and heat, for the six compounds of interest. Future use of the theoretically derived steric energy calculations of new 1*H*-4-substituted imidazole HA $H_3$-selective compounds could expedite the development of experimentally modified derivatization conditions for dabsyl chloride and possibly for other chromophoric derivatizing agents. Further, it would aid in the prediction of producing, and trying to quantify, a heterogeneous solution of mono and di-substituted conjugates.

For the derivatization of compounds, 25 μl of a 50 m*M* $Na_2CO_3$–$NaHCO_2$ buffer solution (pH=9) was added to the acetone solution immediately after extraction. Then 500 μl of a 3.5 m*M* (1.1 mg/ml in acetone) dabsyl chloride solution was added to a vial containing the 1-ml acetone–buffer solution. The vial was then sealed and placed into an 80°C oven to incubate for 20 min for compound I, compound II, and compound IV or 40 min for compound VI, compound V, and compound III. After incubation, the vial was opened and placed back in the oven until complete dryness. To the vial, 300 μl of mobile phase was added in addition to 100 μl of acetone. Samples were vortexed to insure all dabsyl residue was completely in solution. Samples were then placed back into an oven for 10 min at 60°C to evaporate the acetone. The remaining 300 μl of mobile phase solution was filtered through a 0.45-μm nylon syringe filter and then loaded onto the autosampler for injection.

### *2.7. Assay validation*

Assay validation was achieved by analyzing the following HPLC system parameters: linearity, selectivity, accuracy, recovery, and precision. All of these parameters were analyzed for statistical significance using linear least squares analysis, one-way ANOVA, and the student *T*-test.

For the assessment of linearity, stock solutions of mobile phase were spiked with 10, 5, 1, 0.5, 0.25, 0.1, 0.01, 0.005, and 0.001 μg/ml of dabsylated compound I. All injections were done in triplicate and the slope, intercept, and correlation coefficient were determined using linear least-squares analysis. From this data, the upper and lower limits of quantitation were determined for the assay.

Selectivity was accomplished by chromatographically analyzing extracted human plasma, rat plasma, and rat cortical tissue samples that contained no drug. Samples were assayed in triplicate and analyzed for any significant interferences (>2% of the LLOQ peak area).

For the assay, precision was determined by analyzing intra-day and inter-day assay variability of compound I. Both intra-day and inter-day assay variation were determined by assaying four different concentrations of 10, 1, 0.1, and 0.01 μg/ml of compound I-spiked rat plasma in triplicate on four separate days. Peak area, peak height, and retention time were averaged and analyzed for variability. Variation was determined as the percentage of the coefficient of variation.

Measurement of recovery was obtained by analyzing extracted rat plasma spiked with a known concentration of drug and comparing it to a mobile phase control solution containing the identical concentration of drug. Recovery estimates were analyzed in triplicate for compound I.

System precision was determined by analyzing

twelve replicate injections of a single concentration of 10 μg/ml of compound I in mobile phase. The variation between twelve samples was averaged and expressed as a percentage of the coefficient of variation. Assay precision was accomplished by analyzing twelve replicate injections from twelve samples of rat plasma spiked with identical amounts of 10 μg/ml of compound I. Similarly, the variation between the twelve samples was averaged and expressed as a percentage of the coefficient of variation.

### *2.8. Dabsylated compound stability*

Determination of compound stability in the mobile phase was accomplished by running a series of tests using stock solutions of mobile phase spiked with dabsylated compound I and dabsylated compound II. Spiked mobile phase solutions were kept in a lighted room at ambient temperature. A concentration of 10 μg/ml was replicated ($n=4$) for compound II and compound I. Spiked samples were assayed at 0, 6, 12, 24, 48, 72 h and 7 days.

## 3. Results and discussion

### *3.1. Chromatography*

Detection wavelength of the dabsylated conjugates was determined by observing an absorbance spectrum, over the range of 190 to 600 nm, of a 0.1 μg/ml solution of dabsylated compound I in mobile-phase. The observed region of maximum absorbance occurred at 479 nm (Fig. 3). Under identical conditions, dabsyl chloride conjugates of compound II, compound IV, compound V, compound III, and compound VI exhibited maximal absorbance at 479 nm (data not shown).

For this assay, it was determined that the use of an internal standard provided accurate quantitation without having to adjust each sample for extraction efficiency or assay recovery. Compound II, the internal standard used, was chosen for it's relative structural similarity and retention time of 25.9 min to the other compounds tested (Table 2). Compound II's retention time is half way between the earliest compound that eluted, mono-substituted dabsyl derivative of compound V, and the last compound that eluted, dabsylated compound III (Fig. 4). Analysis of drug-free rat cortical homogenate, rat plasma, and human plasma failed to show any evidence of significant exogenous peak interferences to the internal standard, the dabsyl derivative of compound II.

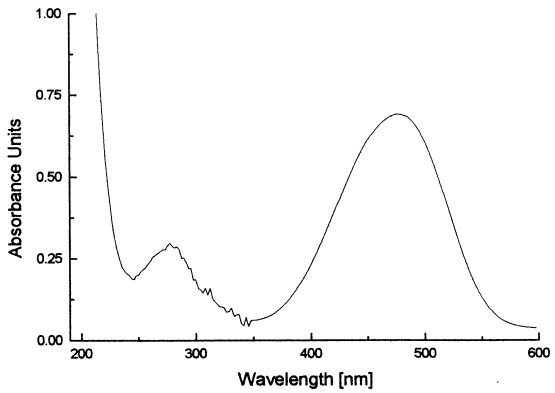


Fig. 3. Spectral absorption profile of dabsylated compound I (1 μg/ml).

It is possible to utilize a variety of isocratic or gradient mobile phase configurations for the elution of any single dabsylated compound. For our purposes, we concluded that an isopropyl alcohol, ACN–TFA, and a sodium acetate buffer system, at a pH of 6.7, was ideal for the elution of the dabsylated HA $H_3$-selective compounds. Through the use of a three-component mobile phase system, in conjunction with an increasing gradient composition over 50 min (Table 1), we were able to individually separate all of the dabsylated $H_3$ antagonists with little or no interference from endogenous peptides and amino acids. Although the data presented here demonstrates the use of a 50-min run-time, routine analytical runs were completed with run-times of 35 min (Table 1). The 50-min run-time was only used for the purpose of separation of all six compounds.

The mono-substituted dabsyl derivative of compound V, the first to elute with a mean retention time of 17.3 min (Table 2), eluted approximately four min after the last major interfering peak in human (data not shown) or rat plasma (Fig. 4) and 1 min after the last significant peak in rat cortical homogenate (data not shown). The other five compounds demonstrated retention times of 4 to 15 min after the elution of the

Table 2
Experimentally derived retention times and theoretical derived log *P* values

| Compound | log *P*±S.D. (Parent compound) | log *P*±S.D. (Derivative) | Retention time (min) (Derivative) |
|---|---|---|---|
| V | 1.64±0.35 | 5.81±0.72[a] | 17.3 |
| VI | 3.26±0.36 | 7.42±0.72 | 24.0 |
| II | 4.36±0.26 | 8.53±0.71 | 25.9 |
| IV | 5.40±0.41 | 8.93±0.75 | 30.4 |
| I | 5.17±0.41 | 9.34±0.69 | 32.5 |
| III | 4.94±0.26 | 9.11±0.69 | 37.7 |

[a] An amide-substitute di-dabsyl derivative can be formed with increased temperature and increased incubation time for compound V. Amide-substituted di-dabsyl compound V conjugate has a log *P* of 9.92+0.93 and a retention time of 41.4 min.

mono-substituted dabsyl derivative of compound V (Fig. 4).

Elution profiles of compound I and compound II in rat plasma (Fig. 5) demonstrate the ease of quantitation of the compounds in a biological matrix. No major interferences (>2% of the LLOQ peak area) were found in any of the drug-free biological matrices (Fig. 5). Human plasma contained the least amount of endogenous peak interferences with two significant peaks at 10.5 and 11.7 min (data not shown). Rat plasma contained several significant peaks all with retention times under 15 min (Fig. 5). Rat cortical homogenate contained numerous significant peaks most of which eluted before 15 min (not shown), although, one significant peak (>2% of the LLOQ peak area) was seen at 16.3 min. Considering that the first dabsylated $H_3$-selective compound to elute is the mono-substituted dabsyl derivative of compound V, with an average retention time of 17.3 min, major peaks from any of the biological matrices

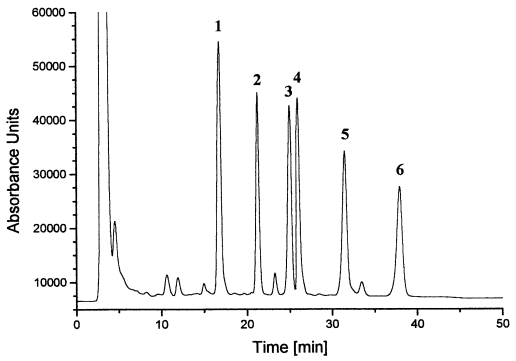


Fig. 4. Typical chromatogram of rat plasma spiked with (1) compound V, (2) compound VI, (3) compound II, (4) compound IV, (5) compound I, and (6) compound III.

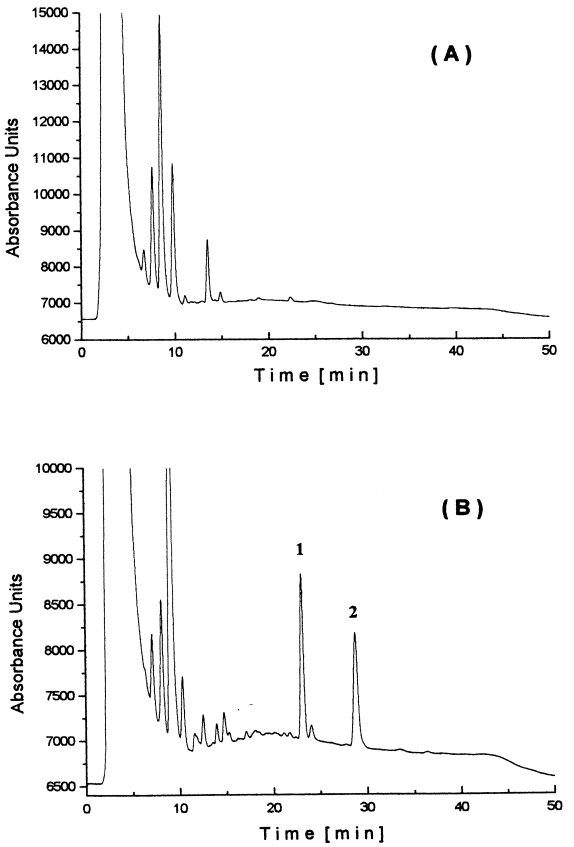


Fig. 5. Typical chromatograms of (A) drug-free extracted rat plasma, (B) compound II (1) spiked at 1 μg/ml and compound I (2) spiked at 1 μg/ml in rat plasma.

examined would not present any interferences to the examined compounds.

### 3.2. Hydrophobicity determinations

Relative hydrophobicities of all parent compounds and their dabsylated conjugates were determined and expressed as the $\log_{10}$ of the calculated water/octanol partition coefficient ($\log P$). Based upon the theoretical $\log P$ values of the uncharged parent compounds and their dabsylated conjugates (Table 2), there was a difference of approximately three-to-four $\log P$ units between the least hydrophobic dabsylated conjugate, compound V, and the most hydrophobic conjugate, compound I. The $\log P$ values were in good agreement with the experimental retention times with a correlation coefficient of 0.9142 (Table 2). Further, based on the $\log P$ of the di-substituted compound V, it was predicted that the retention time would be in excess of 40 min. Experimentally derived retention times for this compound were in good agreement with the theoretically derived retention time.

### 3.3. Compound extraction

Compounds were initially extracted from plasma and homogenized tissues using a liquid–liquid extraction method with moderate success. The liquid–liquid extraction method provided typical recoveries of 75 to 80% of the spiked compound (data not shown). By developing a solid-phase extraction method, we were able to increase the average amount of spiked compound I recovered to 97.2% (Table 3). The solid-phase extraction method was developed utilizing Oasis hydrophilic–lipophilic-balanced extraction cartridges which significantly retained more compound than $C_{18}$-extraction cartridges (85–90% recovery rates, data not shown). We determined that acetone provided an ideal solvent for the elution of the compounds off the Oasis hydrophilic–lipophilic-balanced solid-phase extraction cartridges while still providing a conducive medium for derivatization.

### 3.4. Steric energy determinations and derivatization conditions

Experimentally determined derivatization conditions in conjunction with theoretically derived minimized steric energy values for dabsylated compounds leads to a plausible method for estimating future dabsyl derivatization conditions for structurally diverse $H_3$ antagonists. From the data, it is demonstrated that the two straight-chain compounds, compound I and compound II, in addition to the piperidine-containing compound IV have relatively low steric energy values when compared to the cyclopropane-containing compounds compound VI, compound V, and compound III (Table 4). There is approximately a six fold increase in the calculated steric energy values between the straight chain and the cyclopropyl derivatives. From this theoretically derived data we estimated that the cyclopropyl

Table 3
System and assay validation for compound I

| Concentration (μg/ml) | Intra-assay[a] | | Inter-assay[b] | | System precision[c] | | Assay precision[d] | | Recovery[e] | |
|---|---|---|---|---|---|---|---|---|---|---|
| | Mean | C.V. | Mean | C.V. | Mean | C.V. | Mean | C.V. | Mean (%) | C.V. |
| 10.0 | 9.99 | 0.24 | 9.99 | 0.24 | 9.99 | 0.11 | 10.01 | 0.34 | 97.4 | 1.3 |
| 1.0 | 1.04 | 1.69 | 1.02 | 3.49 | – | – | – | – | 102.3 | 3.4 |
| 0.1 | 0.098 | 2.74 | 0.105 | 5.50 | – | – | – | – | 92.5 | 5.89 |
| 0.01 | 0.0095 | 15.8 | 0.0108 | 10.23 | – | – | – | – | 96.5 | 13.7 |

Rat plasma was pooled and spiked with HLQ, two intermediate concentrations, and LLQ with appropriate compound.
C.V.=Coefficient of variation expressed as a percent.
[a] Determination of intra-assay precision based on $n=3$.
[b] Determination of inter-assay precision based on $n=4$.
[c] Determination of system precision based on $n=12$.
[d] Determination of assay precision based on $n=12$.
[e] Determinations of recovery values based on $n=4$.

Table 4
Theoretical steric energy determinations of parent compounds and their dabsylated derivatives

| Compound | Parent (kcal/mol) | Mono-substituted (kcal/mol) | Di-substituted (kcal/mol) | Δ in steric energy (kcal/mol) |
|---|---|---|---|---|
| I | 22.9934 | 23.6784 | – | 0.6850 |
| II | 22.3082 | 20.4022 | – | −1.906 |
| III | 134.0528 | 134.8887 | – | 0.8359 |
| IV | 36.7938 | 36.8932 | – | −0.0994 |
| V | 127.626 | 126.5603[a] | 138.0367 | −1.0657[b] |
| VI | 125.5512 | 123.0421 | – | −2.5091 |

[a] Represents the single-point steric energy calculation of the *N*-1 substituted compound. The single-point steric energy calculation for the mono-dabsylated amide-substituted compound was determined to be 128.2172 kcal/mol. Thereby demonstrating the preferential selection of the *N*-1 position for dabsyl chloride derivatization.
[b] The change in steric energy of the parent compound and the amide-substituted di-dabsyl compound was determined to be 10.4107 kcal/mol.

analogs will require increased incubation times, and/or higher temperatures, to overcome the increase in the steric energy field. This is supported by our findings that an additional 20-min incubation time was needed to completely derivatize all cyclopropane-containing analogs (Fig. 6).

Through the use of the single-point steric energy determinations, we were able to surmise that compound V would not become a di-substituted compound under the current conditions used to derivatize compounds I through VI at the *N*-1 position on the imidazole head group. Further, compound V required upwards of 2 h for the formation of the amide-substituted di-dabsylated compound (data not shown) versus 40 min for the selective derivatization of the *N*-1 position. This is in agreement with the calculated single-point steric energy determinations of the mono-substituted and the di-substituted compounds (Table 4).

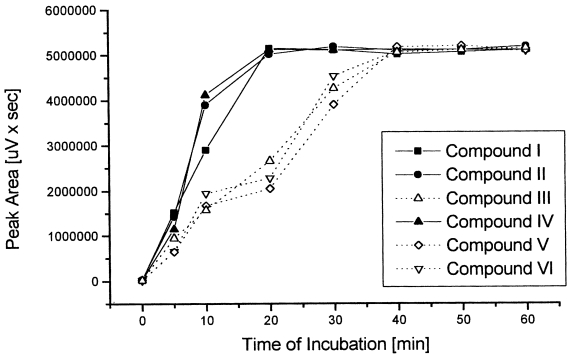


Fig. 6. Graph of dabsyl derivatization versus time of noncyclopropane-containing compounds I, II, and IV and of cyclopropane-containing compounds III, V, and VI.

### 3.5. Assay validation

Linearity of the assay was calculated by analyzing concentrations of 10, 1, 0.5, 0.1, 0.01, 0.005, and 0.001 μg/ml of dabsylated compound I conjugate and compound II conjugate in mobile phase. The mean correlation coefficient of compound I for the six runs was 0.9999 with a coefficient of variation of 0.003% (Table 5). While the calculated value of the intercept was not significantly different than zero. The mean correlation coefficient for compound II for six runs were somewhat less precise at 0.9966 with a

Table 5
Linearity results for compound I and compound II

| Compound[a] | $R^2$ | | *Y*-intercept | | Slope | |
|---|---|---|---|---|---|---|
| | Mean | C.V. | Mean | C.V. | Mean | C.V. |
| I | 0.9999 | 0.003 | 14870.5 | 12.6 | 501524.9 | 1.6 |
| II | 0.9966 | 0.015 | −9931.3 | 18.9 | 581691.6 | 2.3 |

[a] The standard curves used to calculate linearity were done as $n=6$ and spiked at 10, 1, 0.5, 0.1, 0.01, 0.005 and 0.001 μg/ml.

coefficient of variation of 0.015% (Table 5). The calculated intercept value was also not significantly different from zero.

Analysis of drug-free rat cortical homogenate, rat plasma, and human plasma also failed to show any evidence of significant exogenous peak interferences for the six compounds tested.

Overall assay precision was determined by analyzing data obtained from four different concentrations of 10, 1, 0.1 and 0.01 μg/ml of spiked rat plasma run in triplicate on four different days. The intra-assay coefficient of variation ranged from 15.8% for 0.01 μg/ml for compound I to 0.24% for 10.0 μg/ml for compound I (Table 3). While inter-assay coefficients of variation ranged from 10.2% for 0.01 μg/ml of compound I to 0.24% for 10.0 μg/ml of compound I (Table 3).

The average compound recovery was determined to be 97.2% for compound I over the range of 10.0, 1.0, 0.1, and 0.01 μg/ml (Table 3). Recovery rates seemed to remain stable throughout the validation process with only significant variations occurring while extracting at concentrations of 0.01 μg/ml. The calculated coefficient of variation for 0.01 μg/ml was 13.7%.

System and assay precision was determined to be acceptable with coefficient of variation values of 0.11% and 0.34% (Table 3), respectively.

### *3.6. Compound stability*

Stability studies demonstrated that dabsylated compounds remained stable in mobile phase over 7 days with no appreciable loss of conjugate at room temperature (data not shown). With a significant increase in heat (>10°C) or a significant increase in pH (>2 pH units) the conjugate is stable for only 24 h before notable degradation occurs (data not shown).

## 4. Conclusions

The intention of developing this assay was to provide a sensitive and versatile analytical methodology to quantify nanomolar concentrations of imidazoyl $H_3$-selective compounds in biological matrices. Presently, we have demonstrated the development of a precise, selective, and sensitive assay for six structurally diverse 1*H*-4-substituted imidazole HA $H_3$-selective compounds in human plasma, rat plasma, and rat tissue homogenates. The 1*H*-4-substituted imidazole compounds can be detected without any significant endogenous peak interferences and with recoveries greater than 90% in all the tested biological matrices. Detection limits of 1 ng/ml and quantitation limits of 10 ng/ml are amenable for use in pharmacokinetic and biodistribution studies. Comparisons of extraction methodologies has shown the improved utility of new hydrophilic–lipophilic-balanced solid-phase extraction cartridges in the analysis of HA $H_3$-selective compounds. Further, we have developed a method to estimate HPLC retention times, utilizing theoretical log *P* values, and estimating dabsyl chloride incubation conditions, utilizing single-point steric energy values of energy minimized stable conformer.

Although the present assay was used to quantify novel compounds produced in our laboratory, the assay is amenable for the quantification of ligands such as thioperamide, iodoproxyphan, and iodophenpropit which are currently being used in the histamine receptor field to elucidate the functional role of the histamine $H_3$ receptor. This assay is also applicable to other $H_3$-selective agents currently in development or in early human clinical trials, such as the (*R*)-α-methylhistamine prodrug BP 2-94 [33]. Further, this assay can be used to quantify a variety of scientifically important endogenous imidazol-containing compounds such as the amino acid histidine, histidine-containing peptides, and the neurotransmitter histamine; hence demonstrating the assay's value and versatility for evaluating a variety of imidazole-containing compounds in addition to structurally diverse 1*H*-4-substituted imidazole HA $H_3$-selective compounds.

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