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Determination of Ro 19-6327 (Lazabemide) in human plasma and urine by gas chromatography–negative chemical ionization mass spectrometry

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Abstract

A sensitive and specific analytical method was developed for determination of Ro 19-6327 (Lazabemide) in human plasma and urine samples to provide pharmacokinetic data from clinical trials. The new method employs a simple liquid–liquid extraction to isolate the drug from biological samples. The extract is reacted to form the trifluoroacetyl derivative of Ro 19-6327 and then analyzed by gas chromatography–negative chemical ionization mass spectrometry (GC–NCIMS). The lower limit of quantitation of the assay is 0.05 ng/ml for plasma and 5.0 ng/ml for urine, based on 1-ml aliquots. No interferences from anticoagulants, collection devices, or endogenous constituents of plasma and urine were observed. Recovery (64.3%), inter-assay precision (<8% R.S.D.), and accuracy (>85%) of the method were considered acceptable. The assay proved reliable enough to be automated for unattended sample analysis of approximately 50 samples daily. In an additional series of tests, Ro 19-6327 was shown to be stable under conditions that might be encountered during the analysis of samples from clinical trials.

1. Introduction

Ro 19-6327 (Lazabemide, I, Fig. 1A) is a potent and specific inhibitor of monoamine oxidase type B which is being studied for the treatment of Parkinson's disease [1–4]. Pharmacokinetic data from clinical studies indicate that elimination of the compound is non-linear and is best described by a Michaelis–Menten model. Co-existing saturable and non-saturable elimination pathways have been hypothesized to explain the complex elimination characteristics [5–7].

Samples from animal studies have been analyzed for I by HPLC methods with either UV or fluorescence detection [8]. These methods were not completely satisfactory because they could be affected by interferences from anticoagulants and endogenous components of plasma. In addition, the limit of quantitation of these methods, 5 and 1 ng/ml respectively, was not adequate to fully investigate the complex pharmacokinetics of I in humans. An assay procedure that could quantitate subnanogram concentrations was needed.

This report describes a gas chromatographic–mass spectrometric method for quantitative determination of I. The compound was predicted to be a good candidate for such a method of analysis because of its thermal stability, volatility, and its simple functional groups which can be

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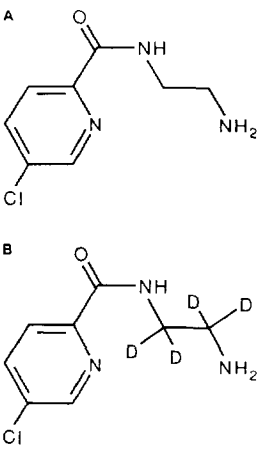

Fig. 1. Chemical structures for (A) Ro 19-6327 (I) and (B) the tetra-deuterated internal standard Ro 19-6327-d_4 (I-d_4).

readily derivatized to form perfluorinated acyl derivatives. These derivatives improve the chromatographic performance of the compound, and their electron-capturing ability under negative chemical ionization conditions greatly enhances the sensitivity of the assay. In this method, I and a tetradeuterated internal standard (I-d_4) are extracted from plasma or urine at basic pH. The organic phase is transferred and evaporated to dryness, and the extract is reacted to form the trifluoroacetyl derivative. The derivatized sample is then analyzed by gas chromatography–negative chemical ionization mass spectrometry (GC–NCIMS).

2. Experimental

2.1. Chemicals

Ro 19-6327 (Fig. 1A) was obtained from the Quality Control Department, Hoffmann-La Roche (Nutley, NJ, USA). Ro 19-6327-d_4 (Fig. 1B) was synthesized by the Isotope Synthesis Group, Hoffmann-La Roche. All solvents were HPLC-grade and were obtained from Fisher Scientific (Fair Lawn, NJ, USA). Potassium hydroxide, ACS grade, was obtained from J.T. Baker (Phillipsburg, NJ, USA). Trifluoroacetic anhydride (TFAA) was obtained from Pierce Chemical Co. (Rockford, IL, USA). Methane (99.99%) and hydrogen (99.999%) were obtained from Liquid Carbonic (Chicago, IL, USA). Distilled water (>18 MΩ cm^2 resistance) was purified with a Milli-Q UF Plus water purification system (Millipore Corp., Bedford, MA, USA).

2.2. Instrumentation and chromatographic conditions

Method development was done on a Kratos MS-890 mass spectrometer with a DS-90 data system, operated in negative-ion mode at 4 kV accelerating voltage. Methane was used as the negative-ion reagent gas. A Hewlett-Packard 5890 gas chromatograph was interfaced to the instrument with a heated transfer line, which allowed the capillary column to be directly introduced to the ion source region. The GC was fitted with a Chrompack glass moving needle injector (part no. 8992) from Chrompack (Raritan, NJ, USA). Chromatography was done with a DB-5 capillary column (15 m × 0.25 mm I.D., 1 μm film thickness) (J&W Scientific, Folsom, CA, USA). The column temperature was 215°C (isothermal), and the injector and transfer line temperatures were 275°C and 235°C, respectively. The carrier gas, hydrogen, was supplied at a column head pressure of approximately 28 kPa (4 psi).

The mass spectrometer was operated at low resolution (ca. 1500 by 10% valley definition) in the selected-ion monitoring (SIM) mode. The ions monitored were m/z 295 (I) and m/z 299 (I-d_4), which were the respective $M^{\cdot -}$ molecular ions of the derivatized compounds.

2.3. Preparation of standards and samples

Stock solutions (1.0 mg/ml) of the hydrochloride salts of I and I-d_4 were prepared in distilled water and stored at 4°C. Serial dilutions of the stock solutions in distilled water provided working solutions for construction of the standard

curves. The concentrations of the working solutions were 1, 2, 10, 20, 100, 400, 1000, 4000, 10 000, and 20 000 ng/ml for I, and 20 and 4000 ng/ml for I-d_4. To construct standard curves, 1-ml aliquots of plasma or urine were spiked with 50 μl of the appropriate working solutions of the analyte and the internal standard. The calibration curve concentrations were 0.05, 0.10, 0.50, 1.0, and 5.0 ng/ml (plasma, low range), 1.0, 5.0, 20.0, 50.0, 200.0, and 500.0 ng/ml (plasma, high range), and 5.0, 20.0, 50.0, 200.0, 500.0, and 1000.0 ng/ml (urine).

Quality assurance (QA) samples were prepared in drug-free plasma or urine, using stock solutions of I prepared separately from those used to construct the standard curves. Plasma QA samples were prepared at concentrations of 10.0 and 300.0 ng/ml and at 0.25 and 2.0 ng/ml for the high and low calibration range standard curves, respectively. Urine QA samples were prepared at concentrations of 50.0 and 500.0 ng/ml. All QA samples were stored at −20°C.

On each day of analysis, duplicate 1.0-ml aliquots of the standard curve samples, QA samples, and drug-free control samples (blanks) were prepared. Experimental samples from study subjects were also pipetted as 1.0-ml aliquots. The internal standard was added to each sample using 50 μl of either the 20 or the 4000 ng/ml working solutions to produce appropriate concentrations for the range of the standard curve. For extraction, 0.2 ml of 0.2 *M* KOH was added to each sample; the samples were then extracted for 10 min with 6 ml of dichloromethane–*n*-pentane–2-propanol (50:50:5, v/v) and centrifuged for 10 min at 1100 *g* and 10°C. The organic layer was transferred to conical-tipped test tubes and evaporated to dryness under nitrogen at 40°C. Next, 200 μl of 1% (v/v) TFAA in anhydrous ethyl acetate was added to each sample, and they were then reacted at room temperature for 60 min to form the trifluoroacetyl derivative. Methanol (100 μl) was added to quench excess reagent, and the samples were evaporated to dryness under nitrogen at 40°C. The residue was dissolved in 50 μl of ethyl acetate for GC–NCIMS analysis. Aliquots of 2 μl were injected.

2.4. Recovery

Compound I was spiked into drug-free human plasma at a concentration of 50.0 ng/ml, in six replicate samples. The samples were extracted as described above, and an equivalent molar amount of the internal standard was added. The samples were then derivatized and analyzed. Recovery was determined from the ratio of the peak heights for I (extracted) and I-d_4 (unextracted) in each sample. The mean recovery from the six replicates, the standard deviation, and the relative standard deviation (R.S.D.) were calculated.

2.5. Sample analysis and calculation

All calculations were based on the ratio of the chromatographic peak height of m/z 295 from I to that of m/z 299 from the internal standard I-d_4. Concentrations of I in experimental and QA samples were determined using an equation established from non-linear isotope dilution regression of the peak-height ratio versus concentration data from the calibration standards. A weighting factor of $1/y^2$ was used in the regression to improve the goodness-of-fit to the data.

Inter-assay precision was assessed from both the calibration standards and the QA samples. On each day of analysis, concentration values for the calibration standards were obtained by back calculation using the regression parameters obtained as described above. A mean value was obtained for the duplicate daily determinations. For each standard, an overall mean value and R.S.D. over 3 or 4 days were calculated. Acceptable inter-assay precision was defined as an R.S.D. for each standard of $\leq 20\%$, and an overall mean R.S.D. of $\leq 10\%$. Inter-assay precision was assessed from the QA samples in a similar manner, with the same definition of acceptable precision.

Accuracy was assessed by determining the percent error in the analysis of QA samples. The theoretical concentration of each QA sample was subtracted from the mean concentration determined over three or four days of analysis. The result was divided by the theoretical value and

converted to a percent. Acceptable accuracy was defined as a percent error of $\leq 20\%$ for each QA concentration, and an overall mean percent error of $\leq 15\%$.

Specificity, i.e. possible interference in the chromatograms from the biological samples or the collection device, was monitored by running control blank samples with each standard curve. In addition, drug-free (zero hour) samples were obtained from patients before they received study drug to screen for interferences from pre-existing medication or other substances.

2.6. Stability of I and I-d_4

The stability of I in plasma was evaluated at room temperature, after storage in a freezer (−70°C), and after cycles of freezing and thawing. For these tests, the compound was spiked into drug-free plasma (50.0 ng/ml). Replicates were stored under the appropriate conditions (or frozen, thawed, and refrozen) for different periods of time and then all analyzed simultaneously.

3. Results

3.1. Chromatography

Direct comparisons were made between the pentafluorobenzoyl, pentafluoropropionic, and trifluoroacetyl derivatives of I. Although each of the three derivatives produced acceptable chromatographic characteristics, the best overall sensitivity for the assay was achieved with the trifluoroacetyl derivative. This was presumably because the TFAA reagent produced the highest yield of derivative.

The mass spectra of the trifluoroacetyl derivatives of I and I-d_4 are dominated by the intense M^- molecular ions. This lack of significant fragmentation enhanced the sensitivity of the assay because minimal ion current was lost through distribution to fragment ions.

During the initial stages of method development, problems were encountered with multiple derivatization of I and I-d_4. In addition to the mono-derivative, di- and tri-trifluoroacetyl derivatives were confirmed by mass spectrometry. This problem was prevented by performing the derivatization at room temperature and by neutralizing excess TFAA with the addition of methanol before the samples were evaporated to dryness.

Figs. 2, 3 and 4 show chromatograms of a control blank (drug-free) plasma sample, a 5.0 ng/ml calibration sample and a 0.05 ng/ml calibration sample, respectively. Trace responses for I and the internal standard were often detected in control plasma samples (Fig. 2) and they were attributable to carry-over in the injection port from previous analyses. This did not interfere with the assay, however, because the intensity was always less than 10% of the low calibration standard. As depicted in Fig. 3, the chromatographic performance was typically affected by nonvolatile constituents in plasma which built up in the injection port, and at the head of the column, causing peak tailing with continued sample analysis. However, with daily cleaning of the injection port, the chromatographic performance never deteriorated to a point which affected the accuracy and precision of the assay procedure. Periodically, it was necessary to maintain the column by rinsing it with *n*-hexane and methanol, or by removing the first meter. These problems also occurred for the analysis of urine samples, but to a much lower extent due to lower concentrations of endogenous material in the extracted samples.

The high sensitivity of the assay is evident from Fig. 4. The response for the 0.05 ng/ml calibration standard typically produced signal-to-noise ratios greater than 5 to 1. Note in Fig. 4 that an unknown endogenous peak was frequently observed which eluted shortly after I.

The typical GC–NCIMS analysis time was 8 min per sample. The chromatogram in Fig. 4 shows a retention time of approximately 2.3 min because the operator chose to use a 4-min solvent delay during this particular study. This figure reflects the output of the Kratos DS-90 data system; the data in Figs. 2, 3 and 5 were produced on the Hewlett-Packard Model 5989A MS-engine, which was the instrument used by

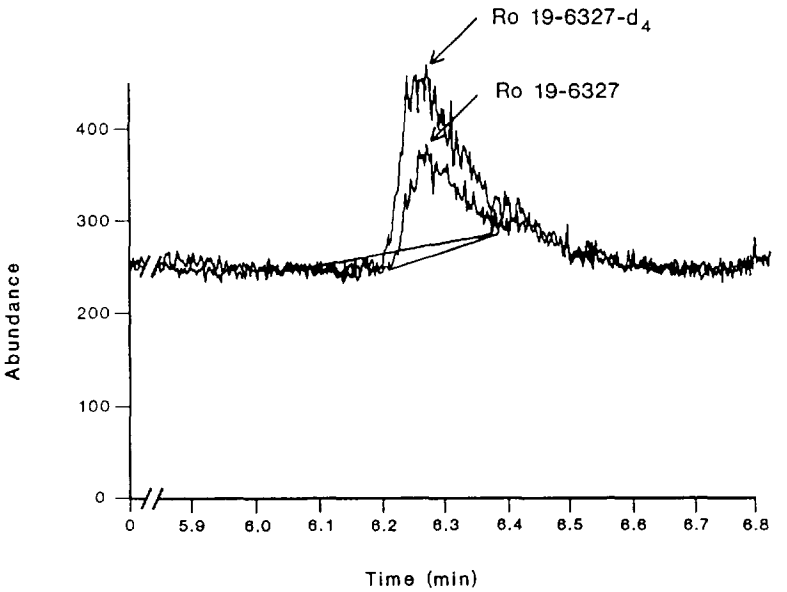

Fig. 2. GC–NCIMS chromatogram of drug-free control human plasma.

the contract laboratory conducting the routine analysis of plasma and urine samples from clinical studies.

An example of sample analysis from study subjects is depicted in Fig. 5. This particular example is from a subject who had been administered I at a dose of 100 mg twice daily for 273 days. The concentration of I was determined to be 395.4 ng/ml plasma, which reflected the steady-state "trough" level of I at time zero, before the morning dose.

3.2. Sensitivity and specificity

Based on a signal-to-noise level of 5 to 1 or greater, and on the requirement that the R.S.D. be less than 20% for the low standard, the limit of quantitation for plasma was established at

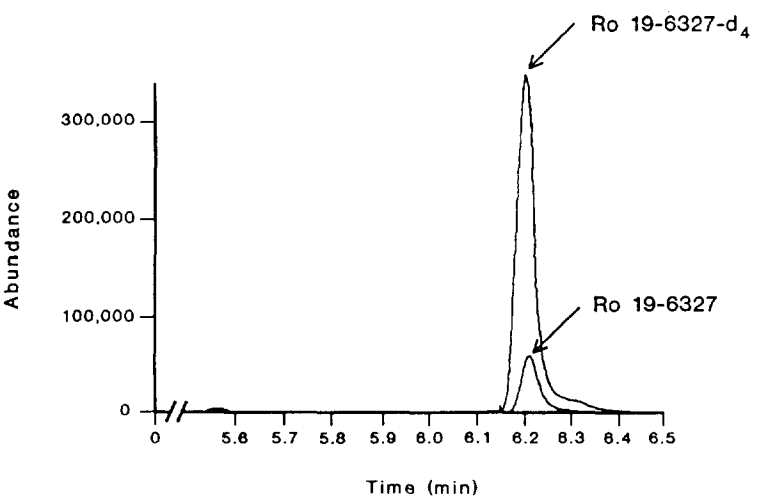

Fig. 3. GC–NCIMS chromatogram of a calibration curve sample (5.0 ng/ml plasma).

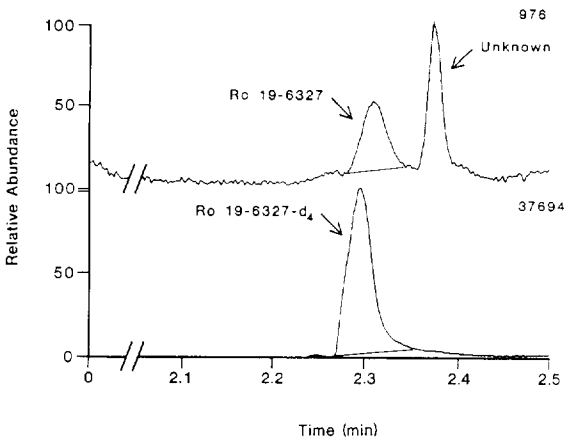

Fig. 4. GC–NCIMS chromatogram of a calibration curve sample (0.05 ng/ml plasma).

0.05 ng/ml. However, this high level of sensitivity has not been required routinely. Quantitation with the high calibration range assay, i.e. limit of quantitation of 1.0 ng/ml, is usually sufficient for most clinical studies. However, when low doses were administered, or when it was necessary to monitor plasma concentrations over extended periods for pharmacokinetic studies, the low calibration range has been readily used. When the assay was applied to urine samples, it was necessary to extend the calibration curve only to 5 ng/ml because of the relatively high concentrations of I found in human urine.

No interferences from anticoagulants have been observed in assays of blood collected in either EDTA or NaF/potassium oxalate Vacutainers. Some lots of control plasma, and experimental samples from some patients, contained endogenous components that eluted very close to the analyte (Fig. 4). These unidentified components, which could cause interference in the low concentration range assay, can be resolved using a 5% phenylmethylpolysiloxane capillary column, such as the J&W Scientific DB-5. The unknown components are difficult to resolve using a dimethylpolysiloxane column.

No interferences from endogenous components or from the collection device (polypropylene bottles) have been observed in assays of urine samples.

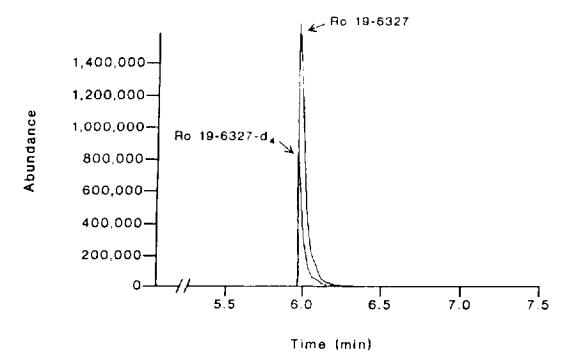

Fig. 5. Example GC–NCIMS chromatogram of a plasma sample taken from a study subject. The concentration was determined to be 395.4 ng/ml.

3.3. Recovery

The recovery of I from plasma was acceptable at 64.3%. The standard deviation from 6 replicate samples was 5.81%, and the %R.S.D. was 9.0%. No attempt was made to increase the recovery because acceptable sensitivity was attainable from the assay with this level of recovery. The recovery of I from urine has not been determined.

3.4. Linearity

The high and low calibration range plasma assays and the urine assay all produced slightly non-linear response–concentration data. The response per ng/ml was typically greater at the low and high extremes of the standard curves than it was through the middle concentrations. This was probably due to adsorption of I in the GC injection port and at the head of the capillary column. At low concentrations, carry-over from previous injections may have contributed somewhat to the response per ng/ml. At high concentrations, the active sites were effectively covered, and a disproportionately high response was again observed. However, isotope dilution regression was found to produce accurate standard curves from the data. Back-calculated con-

Table 1
Standard curve results for three validation trays of the high calibration range plasma assay

	Standard concentration (ng/ml)					
	1.0	5.0	20.0	50.0	200.0	500.0
1st Tray	1.00	5.13	18.47	55.04	203.19	494.12
2nd Tray	1.00	4.95	19.46	52.09	190.46	484.41
3rd Tray	0.99	4.72	19.88	51.29	192.37	464.34
Mean	1.00	4.93	19.27	52.81	195.34	480.96
S.D.	0.006	0.206	0.724	1.975	6.865	15.187
%R.S.D.	0.6	4.2	3.8	3.7	3.5	3.2

centrations of the standard curve points always averaged within 10% error from their theoretical concentrations.

3.5. Precision and accuracy

Tables 1 and 2 show the inter-assay precision calculated for the high and low calibration range plasma assays, respectively. For each assay, the individual %R.S.D. did not exceed 6% on any calibration point. The mean %R.S.D. for each assay was less than 5%. Although not shown, the standard curve results for analysis of urine samples were similar, with the %R.S.D. ranging from 1.8 to 7.8% for individual calibration points. Table 3 shows the inter-assay precision calculated from QA samples. The %R.S.D. did not exceed 8% for any QA sample. These data indicated that the inter-assay precision was acceptable.

The accuracy of the assay procedure as assessed from the QA data is also presented in Table 3. The accuracy of the high calibration range plasma assay was acceptable with errors of +6.8% and −7.5%. However, the accuracy of the low calibration range plasma assay was lower with errors of +13.2% and +15.0%. Inaccuracy in producing the QA samples was the suspected cause of this problem. The accuracy of the urine assay was acceptable with errors of +6.5% and −4.4%.

Table 2
Standard curve results for three validation trays of the low calibration range plasma assay

	Standard concentration (ng/ml)				
	0.5	0.10	0.50	1.00	5.00
1st Tray	0.048	0.12	0.42	1.11	4.99
2nd Tray	0.050	0.11	0.42	1.11	4.99
3rd Tray	0.049	0.11	0.44	1.08	4.99
Mean	0.049	0.11	0.43	1.10	4.99
S.D.	0.003	0.004	0.01	0.02	0.04
%R.S.D.	5.9	3.5	2.4	1.5	0.8

3.6. Stability

The mean concentration of I did not change substantially when plasma samples were stored at room temperature for up to 24 h. The difference from control after 3, 6, and 24 h at room temperature was 3.26, 2.07, and 2.74%, respectively.

In contrast, the concentration of I appeared to increase in plasma samples stored at −70°C. The difference from control after 24 h and 14 days of storage was 3.12 and 9.97%, respectively. Because the experiment involved fortifying control plasma, this unusual change in concentration could not have occurred from the breakdown of metabolites. Therefore, the major contributing factor was probably improper storage of the samples in the freezer. Desiccation may have occurred at −70°C, concentrating the samples.

Table 3
QA results for replicate validation trays of the low and high calibration range plasma assays and the urine assay

	Plasma QA (ng/ml)				Urine QA (ng/ml)	
	0.25	2.0	10.0	300.0	50.0	500.0
1st Tray	0.268	2.29	9.95	286.90	53.95	500.10
2nd Tray	0.280	2.28	11.33	270.66	49.41	436.13
3rd Tray	0.302	2.33	10.77	274.84	58.18	478.65
4th Tray	–	–	–	–	51.54	496.87
Mean	0.283	2.30	10.68	277.47	53.27	477.94
S.D.	0.017	0.03	0.694	8.433	3.762	29.428
%R.S.D.	6.1	1.2	6.5	3.0	7.1	6.2
%Error	+13.2	+15.0	+6.8	−7.5	+6.5	−4.4

When the stability of I at concentrations of 5, 50 and 500 ng/ml in citrated human plasma was investigated, the concentrations changed by −4.0%, −3.4%, and −0.9%, respectively, after 3 months of storage at −20°C [8].

The stability of I to five cycles of daily freezing and thawing was also assessed. There was an apparent increase in concentration of 10.91% with the first freeze–thaw cycle. The concentration did not increase further with additional cycles, but the initial increase was maintained (difference from control, 5.61% to 8.01%). As discussed above, this increase cannot be attributed to instability of metabolites, but it could be caused by experimental error or desiccation of the samples. However, the reason for the apparent increase in the concentration of I with repeated freezing and thawing is currently unexplained.

4. Discussion

This assay has been used successfully with three different mass spectrometers. It was developed and first used on a magnetic sector instrument (Kratos MS-890), using an HP 5890 GC and a moving needle injector. The procedure proved reliable enough to be automated for unattended sample analysis on a quadrupole instrument (Hewlett-Packard Model 5989A MS-engine), using an HP-5890 Series II GC with a split/splitless injector. No deterioration in the performance or sensitivity of the assay was noted. In addition, the assay has been implemented on a Finnigan 3200 quadrupole mass spectrometer, which used an automated HP-5890 Series II GC with a split/splitless injector. When the assay was automated for analysis of a large number of samples each day, it was necessary to adjust the GC temperature program from isothermal operation to include a short temperature program. This helped to elute less volatile sample constituents and, therefore, prolong column performance. It also improved the chromatographic performance of the compounds. Autosampler trays of approximately 50 experimental samples, plus standards, blanks, and QA samples, can be routinely analyzed each day.

Carry-over of I or I-d_4 in the GC injection port or at the head of the column can be minimized by cleaning the injection port regularly and, if necessary, removing the first 0.5–1.0 meter of the capillary column. Avoiding the use of concentrated solutions of I to tune the mass spectrometer is also advisable. Pre-assay "conditioning" of the injection port is not required for this assay.

The major metabolite of I has been shown to be a carboxylic acid compound which results from oxidative deamination of the primary amine. The compound is not believed to be pharmacologically active. This assay procedure was not designed to quantitate this metabolite,

and it does not cause interference. The recovery of the metabolite from plasma at the elevated pH used for the extraction of I is very poor. Furthermore, the compound does not derivatize with TFAA, and without derivatization, it does not chromatograph under the GC conditions used for this assay procedure.

5. Conclusions

The GC–NCIMS assay developed for determination of I in human plasma and urine samples is sensitive, specific, and versatile. Sample preparation is simple and convenient, with only a single liquid–liquid extraction required. Recovery and precision of the method are acceptable to meet the goals of the assay procedure. Although the assay produced standard curves that were slightly non-linear, this was not of concern because the data were reproducible from day to day and the accuracy of the assay was acceptable. To date, this assay has been used to quantitate over 3000 clinical plasma and urine samples.

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