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Hydrogen/deuterium exchange on aromatic rings during atmospheric pressure chemical ionization mass spectrometry

Noel W. Davies[1]*, Jason A. Smith[2], Peter P. Molesworth[2] and John J. Ross[3]

[1]Central Science Laboratory, Bag 74, University of Tasmania, Hobart, Tasmania, Australia 7001
[2]School of Chemistry, Bag 75, University of Tasmania, Hobart, Tasmania, Australia 7001
[3]School of Plant Science, Bag 55, University of Tasmania, Hobart, Tasmania, Australia 7001

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It has been demonstrated that substituted indoles fully labelled with deuterium on the aromatic ring can undergo substantial exchange back to partial and even fully protonated forms during atmospheric pressure chemical ionisation (APCI) liquid chromatography/mass spectrometry (LC/MS). The degree of this exchange was strongly dependent on the absolute quantity of analyte, the APCI desolvation temperature, the nature of the mobile phase, the mobile phase flow rate and the instrument used. Hydrogen/deuterium (H/D) exchange on several other aromatic ring systems during APCI LC/MS was either undetectable (nitrobenzene, aniline) or extremely small (acetanilide) compared to the effect observed for substituted indoles. This observation has major implications for quantitative assays using deuterium-labelled internal standards and for the detection of deuterium-labelled products from isotopically labelled feeding experiments where there is a risk of back exchange to the protonated form during the analysis. Copyright © 2010 John Wiley & Sons, Ltd.

The use of stable isotope dilution mass spectrometry forms the basis of many quantitative analytical methods. As a subset of these, compounds with deuterium replacing hydrogen on an aromatic ring have been used together with liquid chromatography/mass spectrometry (LC/MS) interfaces that use heating during nebulisation and ionisation, such as Atmospheric Pressure Chemical Ionisation (APCI) and Turbo Ion Spray. Examples include amphetamines,[1] nicotine and its metabolites,[2–4] indoles,[5] azo dyes[6] and benzodiazepines,[7] among others. As well as their common use as internal standards for quantitative analyses, deuterated compounds are also the target analytes in many biosynthetic studies (e.g. [8–10]), and in drug metabolism and disposition studies using labelled pharmaceuticals.[11] The synthesis of deuterium-labelled compounds for use in both mass spectrometry and fundamental studies by deuterium/hydrogen (D/H) exchange from the unlabelled versions has been comprehensively reviewed.[12] Common methods include acid- and base-catalysed exchange, as well as the use of homogeneous and heterogeneous catalysts.

As part of a compound-based approach to determining hormone pathways in plants, and the effects of specific genes on these pathways, we have used acid-catalysed D/H exchange to synthesise several stable-isotope-labelled plant hormones or their putative precursors for use as internal standards. These standards are typically prepared from the corresponding unlabelled compounds by treatment with a solution of deuterium chloride for days at room temperature or hours at 100°C in a sealed tube using a microwave reactor. Several deuterated indole-ring plant hormones and their precursors prepared this way have been successfully utilised by our group in quantitative gas chromatography (GC)/MS determinations.[9] In these compounds deuterium replaces all hydrogens attached to the indole ring.

We recently attempted APCI LC/MS determinations at the low to sub-nanogram range for tryptamine (1a – molecular weight (MW) 160 Da) and Nω-acetyltryptamine (2a – MW 202 Da) utilising the nominally 2,4,5,6,7-D_5-labelled versions of each analyte (1b & 2b), respectively, as internal standards. However, significant amounts of the D_4, D_3, D_2, D_1 and even D_0 forms of these substituted indoles were unexpectedly detected. Investigations by other techniques (GC/MS and electrospray (ES)-MS) confirmed that the compounds themselves had not lost any deuterium atoms, and revealed that this effect was occurring during the APCI desolvation/ionisation process.

Many MS techniques, including APCI, have been used as tools to investigate conventional 'exchangeable' protons, such as those on hydroxyl and amino groups, by infusing the ion source with D_2O or CD_3OD[13,14] and following the D/H exchange occurring in the gas phase. However, we are unaware of any reports on the acid-catalysed gas-phase exchange of aromatic protons/deuterons during LC/MS analysis. This has potentially significant implications for

*Correspondence to: N. W. Davies, Central Science Laboratory, Bag 74, University of Tasmania, Hobart, Tasmania, Australia 7001.
E-mail: noel.davies@utas.edu.au

analyses where deuterated aromatic ring compounds are either used as internal standards or are themselves the target analytes.

Parameters such as sample concentration, nature of the mobile phase, flow rate, and APCI desolvation temperature were examined for their influence on the magnitude of this effect. The effect of chromatographic peak tailing on H/D ratios was also examined.

EXPERIMENTAL

Preparation of labelled standards

The labelled indole compounds were prepared as described previously.[9]

2,3,4,5,6-D_5-Aniline (3a)

D_5-Nitrobenzene (100 μL, 0.978 mmol; Cambridge Isotope Laboratories) and zinc (710 mg, 10.8 mmol) were refluxed in glacial acetic acid (1 mL) for 1 h. The residue was diluted with water (5 mL) and poured into 2M sodium carbonate solution (20 mL). The zinc salts were dissolved with conc. ammonia, and extracted with *tert*-butyl methyl ether (3 × 15 mL). The combined organic extracts were washed with water (20 mL), brine (20 mL), dried on sodium sulphate, filtered and the solvent removed under reduced pressure, yielding the title compound (64.2 mg, 0.655 mmol) as a pale oil in 67% yield.

2,3,4,5,6-D_5-Acetanilide (3b)

Triethylamine (1.10 mL, 7.92 mmol), acetic anhydride (0.620 mL, 6.58 mmol) and dimethylaminopyridine (22.3 mg, 0.183 mmol) were added sequentially to a solution of 2,3,4,5,6-D_5-aniline (64.0 mg, 0.654 mmol) in anhydrous dichloromethane (5 mL) under an atmosphere of nitrogen. After stirring for 18 h the reaction was diluted with dichloromethane (20 mL), and the organic phase washed with saturated potassium hydrogen sulphate solution (2 × 20 mL), 2 M sodium carbonate solution (2 × 20 mL) and dried with anhydrous magnesium sulphate. The mixture was filtered through a plug of silica gel using 60% ethyl acetate/hexanes for elution. The solvent was removed under reduced pressure yielding the title compound (58.7 mg, 0.419 mmol) in 64% yield.

Derivatisation and GC/MS analysis

2,4,5,6,7-D_5-Tryptamine (1b) was converted into its bis-pentafluoropropionyl derivative and analysed by GC/MS as described previously[9] using a Varian 1200 triple quadrupole mass spectrometer in tandem mass spectrometry (MS/MS) mode. Corrections were made for natural isotopes and the 0.4% interference between adjacent channels on the quadrupole SRM analyses (as determined by analysis of unlabelled tryptamine) before calculating the degree of deuterium incorporation. D_5-Acetanilide was analysed directly by GC/MS on a Kratos Concept ISQ in selected ion monitoring mode with channels for D_0 through to D_7, at a resolution of 1000, and dwell time of 50 ms per ion. Appropriate corrections were made for naturally occurring isotopic abundances.

APCI-MS analysis

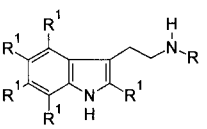
Initial APCI-MS analyses were undertaken on a Finnigan (Thermo) LCQ Classic fitted with a 5 μL Cheminert loop valve for sample introduction. Organic solvents were HPLC grade; water was Milli-Q. Solvents were pumped by a Waters Alliance 2690 HPLC. Default conditions were: spray current 6 μA, capillary voltage 15 V, capillary temperature 150°C, desolvation temperature 350°C, mobile phase 50:50 methanol/1% acetic acid in water, flow rate 0.5 mL/min and sheath gas pressure 40 psi. These parameters were varied for individual experiments as described below. Tryptamine, aniline and acetanilide were analysed as their $[M+H]^+$ ions, and nitrobenzene as the $[M]^-$ ion. For all data any traces of background ions at the relevant *m/z* values were removed by baseline subtraction prior to measurement of relative abundances. Samples were dissolved in methanol and injected via the loop into the mobile phase stream. Narrow scan ranges were employed, including 'Zoomscan' higher resolution mode where possible.

Electrospray MS analysis

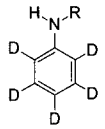
Electrospray analyses were carried out on the LCQ at a capillary temperature of 200°C, capillary voltage of 15 V, capillary temperature 200°C, needle voltage of 4 kV and sheath gas pressure of 85 psi.

Orbitrap APCI-MS

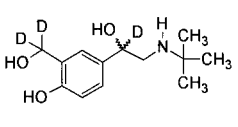
Further APCI analyses were carried out on a Thermo Scientific Orbitrap FTMS mass spectrometer by loop injection (2 μL loop) into a stream of 0.5 mL/min of 50:50 methanol/1% acetic acid. The spray current was 3.6 μA, capillary voltage 3 V, capillary temperature 200°C, desolvation temperature 350°C, and sheath gas pressure 30 psi. Resolution was set to 30 000. Narrow scan ranges around the ions of interest were used.



1a R, R^1 =H
1b R =H, R^1 =D
2a R= $COCH_3$, R^1 =H
2b R= $COCH_3$, R^1 =D



3a R=H
3b R= $COCH_3$



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RESULTS

The level of deuterium incorporation into the D_5-tryptamine (1b) standard, as determined by GC/MS as its bis-pentafluoropropionyl derivative (after appropriate corrections for naturally occurring isotopes and the slight inter-channel interference between adjacent masses on the quadrupole system used), was 94 atom%, with 74% being in the D_5 form and no detectable D_1 or D_0 (see Table 1).

As noted, initial attempts at APCI LC/MS using this D_5-tryptamine (1b) sample yielded mixed levels of deuteration. The $[M+H]^+$ ion for D_5-tryptamine (1b) is at *m/z* 166, and other degrees of deuteration were clearly observed at *m/z* 165 (D_4), 164 (D_3), 163 (D_2), 162 (D_1) and finally the D_0 form at *m/z* 161. Blank runs after tryptamine injections did result in the observation of tryptamine ions due to the surface interactions with the loop and transfer lines, and so several wash cycles of the loop with 1% acetic acid were employed to remove this 'memory' effect. After this procedure no significant ions

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Table 1. Percentage of each level of deuteration and total aromatic atom% deuterium of nominally 'D_5'-tryptamine (1b) observed under different analytical conditions

Experiment	%D_5	%D_4	%D_3	%D_2	%D_1	%D_0	Atom% D
GC/MS (single analysis)	74.3	20.2	4.8	0.6	nd	nd	93.7
LCQ Electrospray	77.1 ± 1.1	17.8 ± 0.8	3.0 ± 0.6	nd	nd	nd	95.0 ± 0.3
APCI acetonitrile	73.4 ± 1.8	17.9 ± 0.4	5.0 ± 0.7	1.8 ± 0.2	0.9 ± 0.1	1.0 ± 0.1	91.6 ± 0.5
APCI methanol	61.8 ± 1.4	25.4 ± 1.1	7.0 ± 0.7	2.9 ± 1.0	1.5 ± 0.3	1.4 ± 0.6	87.8 ± 1.2
APCI Orbitrap FTMS 2 ng	60.3 ± 2.1	20.8 ± 0.1	6.0 ± 0.5	3.2 ± 0.4	2.5 ± 0.5	7.2 ± 0.6	82.3 ± 0.5
APCI aq/MeOH 50 ng	47.6 ± 0.7	31.7 ± 0.2	11.6 ± 0.2	4.8 ± 0.3	2.6 ± 0.3	1.8 ± 0.3	82.3 ± 0.6
APCI Aq/MeOH*	38.6 ± 0.6	26.6 ± 1.0	12.9 ± 0.3	8.7 ± 0.8	6.7 ± 0.1	6.4 ± 0.7	72.4 ± 0.2
APCI Aq	32.6 ± 0.8	29.5 ± 1.3	15.9 ± 1.3	9.5 ± 0.8	7.1 ± 0.4	5.4 ± 0.5	70.9 ± 1.1
APCI Aq/MeOH 0.2 mL/min	27.0 ± 0.3	29.2 ± 1.3	14.2 ± 0.8	11.0 ± 0.3	8.7 ± 1.0	10.0 ± 1.1	65.0 ± 1.3
APCI Aq/MeOH 1 ng	28.4 ± 1.7	25.4 ± 2.1	13.7 ± 0.8	10.5 ± 1.1	10.2 ± 1.4	11.8 ± 1.8	63.2 ± 1.0

*This experiment was repeated for each variable tested; this row relates to the testing of different mobile phases.
Averages are shown with standard deviations; n = 3. APCI analyses were all 5 ng injections on the LCQ, using desolvation temperature of 350°C and flow rate of 0.5 mL/min unless otherwise stated. 'Aq' is 1% acetic acid in water. 'Aq/MeOH' is 50:50 1% acetic acid in water/methanol. 'nd' is not detected. Appropriate corrections were made for natural isotope abundances. Within experimental error, the GC/MS and electrospray data effectively both represent the actual deuterium labelling of the sample.

were observed at any of the target *m/z* values for blank injections.

Figure 1 shows the marked effect of APCI desolvation temperature, with substantial increases in H/D exchange at 350°C (the optimum temperature for this analysis) compared to 250°C. Due to the significant effect on yield of protonated molecules by working significantly below or above optimal desolvation temperatures, only these two desolvation temperatures were examined quantitatively.

Figure 2 shows the effect of various pure and mixed mobile phases. Very little exchange was noted with acetonitrile, small reductions in atom% deuterium were observed with methanol, and the most exchange was seen with 100% aqueous mobile phase. The presence of 1% acetic acid in the mobile phase had little apparent effect on the level of H/D exchange, since H_3O^+ is efficiently generated by the APCI discharge voltage.

The degree of H/D exchange was strongly affected by the amount of sample injected, as noted by the difference between 1 ng, 5 ng and 50 ng loop injections (Fig. 3) with very marked increases in exchange at the lower levels. This was most apparent in terms of percentage of D_0, which more than tripled between the 50 ng and 5 ng levels. Moreover, as tryptamine tends to 'tail' due to surface binding even on loop injections, a strong discrimination was observed across the peak, with dramatically increased H/D exchange observed on the tail of the peak as the sample concentration dropped. Figure 4 shows this effect by examining the mass spectra averaged across the peak at half height and across the tail of a loop injection, with markedly lower atom% deuterium seen

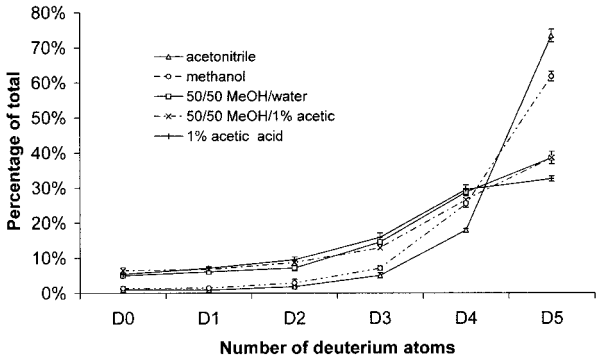

Figure 2. Effect of APCI mobile phase on H/D exchange for nominally D_5-tryptamine (1b). Flow rate 0.5 mL/min, APCI desolvation temperature 350°C, 5 ng injections. Each point is the average of three loop injections – error bars show standard deviations.

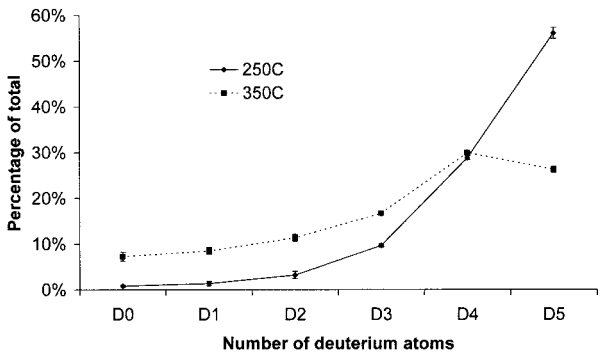

Figure 1. Effect of APCI desolvation temperature on H/D exchange for nominally D_5-tryptamine (1b). Flow rate 0.5 mL/min, mobile phase 50:50 methanol/1% acetic acid, 5 ng injections. Each point is the average of three loop injections – error bars show standard deviations.

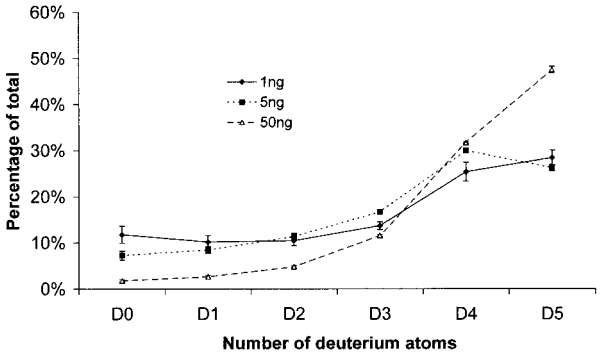

Figure 3. Effect of amount of nominally D_5-tryptamine (1b) injected. Flow rate 0.5 mL/min, APCI desolvation temperature 350°C, mobile phase 50:50 methanol/1% acetic acid. Each point is the average of three loop injections – error bars show standard deviations.

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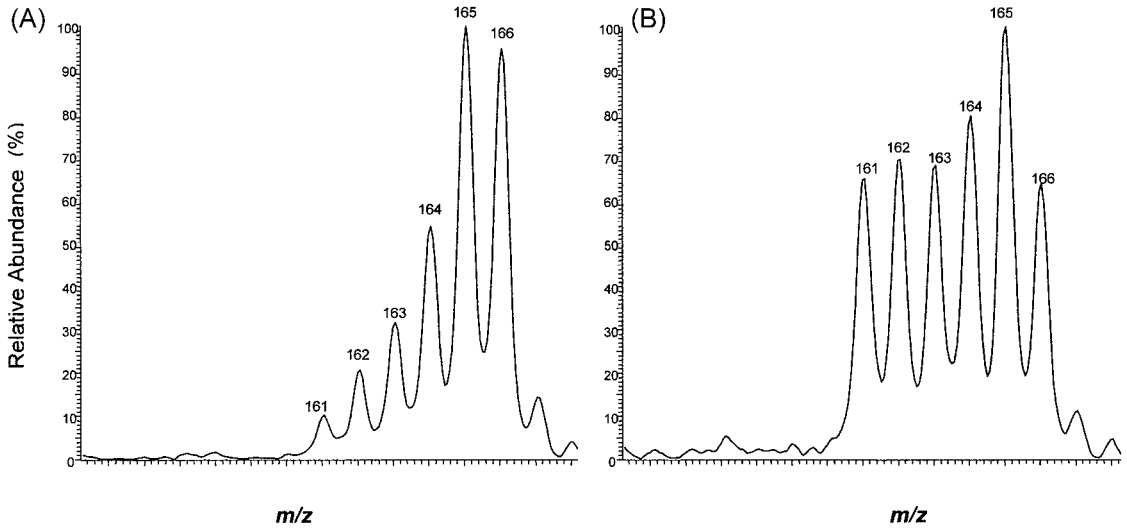

Figure 4. Mass spectra in the protonated molecule region for nominally D_5-tryptamine (1b) under APCI conditions for a 5 ng injection with water present in the mobile phase: (A) average across the peak at half height and (B) average towards the tail of the loop injection peak.

in the latter. The effect can also easily be observed by examining mass chromatograms at each level of deuterium incorporation. Figure 5 shows the effect on the measurement of deuterium incorporation by inclusion and exclusion respectively of the chromatographic peak tailing.

There was an inverse correlation between flow rate and H/D exchange (Fig. 6), which can be readily attributed to the lower flux of sample through the ion source at lower flow rates reducing the sample concentration in the ion source and hence increasing H/D exchange.

To eliminate the possibility of the observed H/D exchange being due to some specific catalytic activity in the 11-year-old ion source used, the same measurements were undertaken on a Thermo Orbitrap mass spectrometer in APCI mode. This also resulted in H/D exchange effects for D_5-tryptamine (1b), although of a substantially lower magnitude using similar conditions to those used on the LCQ, indicating that source design and/or history of use are significant factors in the degree of exchange observed. A 2 ng injection of D_5-tryptamine (1b) on the Orbitrap yielded the same atom% deuterium as a 50 ng injection on the LCQ, but the former had unexpectedly elevated levels of D_0 (7.2%) relative to the other partially deuterated forms. All Orbitrap data were acquired at high mass resolution (30 000) and the quantitation was done on the exact masses for the various tryptamine $[M+H]^+$ ions, minimising the possibility of interferences from background ions.

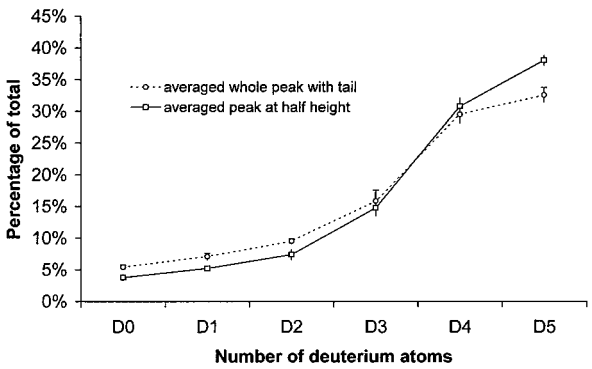

Figure 5. Effect of peak tailing on deuterated tryptamine measurements. Solid line is average across the peak at half height, dotted line is average across the whole peak. APCI desolvation temperature 350°C, mobile phase 50:50 methanol/1% acetic acid, 5 ng injections. Each point is the average of three loop injections – error bars show standard deviations.

Table 1 summarises the change in deuterium levels for D_5-tryptamine (1b) under some of these different conditions and on two different instruments. Other indole compounds, including D_5-Nω-acetyltryptamine (2b), showed very similar levels of H/D exchange. The GC/MS and ES-MS results indicated some D_4, D_3 and a trace of D_2 forms were present in the original sample – these data then represent the 'no exchange' case.

D_5-Nitrobenzene (99.6% atom% D; Cambridge Isotopes laboratories Inc.) had poor ionisation efficiency in negative APCI, and at the relatively high concentrations for which the M^- ion could be clearly observed there was no detectable H/D exchange. Despite excellent sensitivity in positive ion APCI, no significant H/D exchange could be detected for D_5-aniline (3a). For D_5-acetanilide (3b) the effect was initially difficult to detect, but when amounts were lowered to single and sub-nanograms a small amount of H/D exchange was noted. However, the atom% deuterium only changed from ~94% for 50 ng injections to ~89% for 1 ng injections. Table 2 shows details for D_5-acetanilide (3b) at different injection levels. Statistical analysis revealed that all differences in atom% deuterium between the 50 ng, 5 ng, and 1 ng levels for D_5-acetanilide were statistically significant ($P < 0.001$ for 50 ng vs. 5 ng, 5 ng vs. 1 ng and 50 ng vs. 1 ng).

Racemic D_3-salbutamol (4), containing no aromatic ring deuterium atoms, unsurprisingly did not show any evidence of H/D exchange down to sub-nanogram amounts under the same APCI conditions as used for D_5-tryptamine (1b).

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Table 2. Percentage of each level of deuteration and total aromatic atom% deuterium of nominally 'D_5'-acetanilide (3b) during APCI

Experiment	%D_5	%D_4	%D_3	%D_2	%D_1	%D_0	Atom% D
GC/MS (single analysis)	71.6	25.5	3.0	nd	nd	nd	93.8
APCI Aq/MeOH 50 ng	73.4 ± 0.6	23.0 ± 0.4	3.3 ± 0.3	0.3 ± 0.05	nd	nd	93.9 ± 0.1
APCI Aq/MeOH 5ng	70.3 ± 0.7	24.9 ± 0.7	4.0 ± 0.2	0.8 ± 0.1	nd	nd	92.9 ± 0.2
APCI Aq/MeOH 1ng	62.4 ± 1.5	25.3 ± 0.3	8.3 ± 0.9	4.1 ± 1.0	nd	nd	89.2 ± 0.8

Averages are shown with standard deviations; n = 4. APCI analyses were conducted using a desolvation temperature of 350°C and a flow rate of 0.5 mL/min. 'Aq/MeOH' is 50:50 1% acetic acid in water/methanol. 'nd' is not detected. Appropriate corrections were made for natural isotope abundances. Differences in atom% D between the 50 ng, 5 ng, and 1 ng injections were all statistically significant ($P < 0.001$). Within experimental error, the GC/MS and 50 ng APCI data effectively both represent the actual deuterium labelling of the sample.

DISCUSSION

A nominally 'D_5'-labelled tryptamine (1b) standard that had been shown by GC/MS analysis to be ~75% D_5 and ~94 atom% deuterium, with no detectable D_0, resulted in as little as 28% D_5 and 63 atom% deuterium, and as much as 12% D_0 upon APCI-MS analysis under conditions that strongly favoured exchange – i.e. a low level of analyte, presence of some aqueous mobile phase and relatively high desolvation temperature. Given that H_3O^+ and/or $CH_3OH_2^+$ were present in abundance in the gas phase whenever significant exchange was detected, and the relatively high desolvation temperatures involved, H/D exchange in principle could be claimed to be not unexpected given that acid-catalysed exchange is the method by which deuterated aromatics can be prepared in the first place.

These solution-phase syntheses of deuterium-labelled compounds involve the electrophilic aromatic substitution of the aromatic ring with deuterium chloride resulting in exchange of the hydrogens for deuterium. However, the apparent efficiency of this back-exchange under APCI conditions when H_3O^+ and/or $CH_3OH_2^+$ were present, given that the solution methods require many hours at high temperatures to achieve high percentage of deuterium incorporation, was quite unexpected. The exposure time to acidic gas-phase cations under APCI was only fractions of a second, and yet the aromatic ring atom% deuterium was reduced by up to one-third under certain conditions. The exchange was even more marked at extremely low sample concentrations, but it was difficult to take quantitative measurements with sufficient precision in this picogram range on a signal distributed among five different ions. Nevertheless this is the range at which many quantitative analyses are required – for plant hormones the initial spike of internal standard to the whole sample is often in the sub-nanogram range.[5] Unsurprisingly, the use of acetonitrile alone as the mobile phase resulted in negligible H/D exchange.

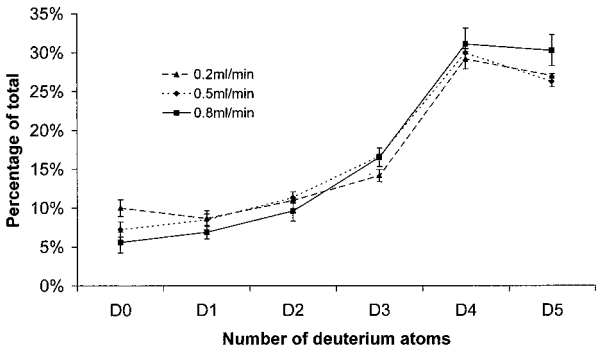

Figure 6. Effect of mobile phase flow rate on H/D exchange for nominally D_5-tryptamine (1b). APCI desolvation temperature 350°C, mobile phase 50:50 methanol/1% acetic acid, 5 ng injections. Each point is the average of three loop injections – error bars show standard deviations.

Strong correlations with all the parameters tested were observed. In addition to the specific parameters investigated, aspects such as source design, discharge current, needle position, source cleanliness and prior history, and inlet capillary temperature may all influence the degree of aromatic H/D exchange.

We suggest that indole compounds are more prone to exchange due to the high electron density of the aromatic ring due to electron donation from the indole nitrogen.[15] This is consistent with the reactivity trend for electrophilic aromatic substitution in which aromatics with electron-donating groups react faster than those with electron-withdrawing groups attached. As a nitro group is strongly electron-withdrawing, it is strongly deactivating for this reaction and hence no exchange is observed. Aniline might be expected to behave the same as indole; however, under the acidic APCI conditions the amine will be protonated and the resultant ammonium ion acts as an electron-withdrawing group. For tryptamine the APCI protonation is on the terminal amine, and hence remote from the indole ring, minimising any electron-withdrawing effect. With acetanilide, protonation of the carbonyl oxygen rather than the nitrogen results in an electron-withdrawing resonant hydroxylated iminium ion, minimising H/D exchange. The result for these compounds is consistent with the reactivity of the different aromatic systems towards electrophilic aromatic substitution under acidic conditions.[15,16] Further evidence for the gas-phase reactivity of indoles can be found from the proton affinities calculated for the carbons at positions 2, 4, 5, 6 and 7, which are all between 876 and 900 kJ mol^{-1}.[17] In comparison the proton affinity of carbons 2 to 5 of nitrobenzene is 676 kJ mol^{-1},[18] while those of water and methanol are 696 and 761 kJ mol^{-1}, respectively.[19]

A significant implication of these observations is that quantitative data may be seriously skewed by the conversion of almost fully deuterated standards of electron-rich aromatics to lower levels of deuterium incorporation if direct isotope dilution calculations are used, as is typically the case in plant hormone studies. Furthermore, the detection of a D_0 peak may not indicate the presence of genuine

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unlabelled material in the original sample. As most targeted quantitative determinations do not include channels for levels of deuterium incorporation less than the nominal level, this H/D exchange might go unnoticed unless sufficient of the D_0 form is created to be detected in negative control samples.

As exchange of deuterium on an aromatic ring can potentially occur under the acidic conditions of the APCI plasma, inclusion of channels for intermediate levels of deuteration for all deuterated standards in initial experiments may be prudent. In this small study the levels of analytes were relatively high due to the need to measure with precision the various partially deuterated forms down to less than 1% of the total signal, and also due to the age of the instrument used for the bulk of the work. Modern mass spectrometers typically have sensitivities in the femtogram range for many analytes, further increasing the need for caution when using deuterated standards with heated API ion sources, as there is a strong inverse correlation between the amount of analyte and the degree of H/D exchange.

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