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Quantitative NMR assay for aspirin, phenacetin, and caffeine mixtures with 1,3,5-trioxane as internal standard

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Abstract: The method for ^{1}H NMR determination of aspirin, phenacetin and caffeine (APC) mixtures has been improved by the use of 1,3,5-trioxane as an internal standard. The trioxane absorption occurs in a peak-free region of the spectrum and produces no interferences with any of the analytes. Compared to the original method with caffeine as an external standard, the present method appears to offer better accuracy and precision. Average errors relative to the correct results were: aspirin, 1.0%; phenacetin, 0.8%; and caffeine 1.8%, for known standard mixtures. Coupling constants, $^{1}J_{^{13}CH}$, were determined for the methyl groups of aspirin and caffeine and for the trioxane methylene group to clarify potential ^{13}C satellite interferences.

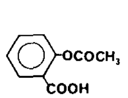
Keywords: *Aspirin; phenacetin; caffeine; quantitative analysis by NMR; 1,3,5-trioxane as internal standard.*

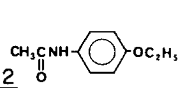
Introduction

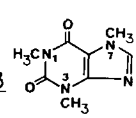
The elegant method of Hollis [1] reported in 1963 for the quantitative analysis of aspirin (**1**), phenacetin (**2**) and caffeine (**3**) (APC) in mixtures represented one of the very early and effective applications of ^{1}H nuclear magnetic resonance (NMR) for pharmaceutical analysis. Simple area integrations of selected resonances of the three compounds provided a direct simultaneous determination of the APC components with a single spectral scan of the mixture. Insoluble excipients, such as starch, did not interfere when $CHCl_3$ or $CDCl_3$ was utilized as solvent. The presence of carbon-13 satellite peaks was also partly taken into consideration.

However, although a single scan of the sample mixture sufficed for measuring relative

Figure 1
Schemes 1–3.

1 

2 

3 

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amounts of the APC constituents, the requirement of an external standard meant that a second scan and integration were also needed, and imposed the problems usually associated with an external standard. Thus, run-to-run variation in the observed spectral or integral amplitudes, the possibility of differences between NMR sample tubes for standard and sample, the irreproducibility of instrument operating parameters, etc. could all contribute to errors in accuracy and precision. Notwithstanding these limitations, the method of Hollis appeared to be excellent.

Only a few assays for APC employing NMR appear to have been reported. Recently, a ^{1}H NMR method was described [2] using an internal standard, piperonal (3,4-methylenedioxybenzaldehyde), which had been redistilled. The integral of the methylene resonance near 6δ was compared to the CH_3-resonance of aspirin at 2.3δ, the CH_3-triplet of phenacetin at 1.3δ, and the caffeine singlet at 3.4δ. (The latter peak has been assigned to the N_1–CH_3 of caffeine in $CDCl_3$ [3]; other workers [4] have also attributed the highest field CH_3-resonance to the N_1–CH_3.) The workers in [2] reported no interference with tablet excipients. Hollis has specifically proposed [1] the rejection of the triplet at 1.3δ as an analytical peak because several commercial APC preparations contained an impurity that produced an absorption peak overlapping the high field component of the triplet. The present results seem to support this. Some analyses of generic APC tablets were found to contain absorptions (from excipients or binders) which overlapped with the upfield branch of the methyl triplet of phenacetin, in agreement with [1], and analytical use of this resonance would be ruled out for such samples. Evidently different APC tablet compositions can account for differing observations [2]. It should also be noted that at 60 MHz the aromatic proton absorptions of piperonal between 6.70 and 7.47δ [5] partly overlap absorptions of the aryl protons of aspirin and phenacetin and come close to overlapping the caffeine peak near 7.51δ. Although these regions are not used for quantitative purposes, use of piperonal would obscure this area and interfere with qualitative analysis. This information, which could be of importance for unknown analyses, as in forensic applications, would be lost. Further, aromatic aldehydes tend to undergo facile air oxidation leading to impurities which could require frequent purifications to avoid contaminants in the internal standard. Finally, piperonal is a low melting solid, m.p. 37°C [6], leading to potential difficulties in handling.

For an alternative APC assay, workers have employed carbon-13 NMR with anisole as internal standard [7]; nonaqueous titration [8]; volumetric or titrimetric methods [9]; column separations and spectrophotometry [10]; computer-controlled ultraviolet (UV) spectrophotometry [11]; gas chromatography (GC) [12]; automated or ion-exchange high-performance liquid chromatography (HPLC) [13]; polystyrene gel micro-HPLC [14]; thin-layer chromatography combined with ancillary methods [15]; and others. In some cases, additional components may also be determined.

The possibility of finding a more suitable internal standard as a further improvement to the ^{1}H NMR assay method has been explored. Such an internal standard should be cheap, readily available in satisfactory purity, with a proton resonance that would not interfere with any of those of the APC components. It should also be relatively stable, free from chemical interaction with the APC mixture or air, and should be solid for ease in weighing. Selection of 1,3,5-trioxane appeared to fulfill these requirements. Its ^{1}H NMR spectrum in $CDCl_3$ at 28° consists of a sharp singlet near 5.15δ, adequately removed from the nearest APC resonances near 4δ (the N_7–methyl of caffeine and the methylene of phenacetin).

Experimental

Methodology

A number of APC determinations using 1,3,5-trioxane as internal standard were carried out by simply weighing the trioxane to an accuracy of ±0.1 mg directly into the NMR sample tube. Alternatively, the trioxane could be prepared as a volumetric solution and transferred to the APC sample with a syringe or micropipette. For smaller samples, a microbalance would clearly prove useful (but was not used for this present work). All spectra were obtained on a Varian EM-360A 60 MHz ^{1}H NMR spectrometer at a probe temperature of 28°C, using deutero-chloroform (99.8 atom % D, Aldrich Chemical Co., Milwaukee, WI) as solvent with tetramethylsilane (TMS) as internal standard for chemical shift. $CDCl_3$ and TMS were dried before use and stored over 3A molecular sieves. Known standard mixtures of aspirin, phenacetin and caffeine were prepared by directly weighing out the reagents to an accuracy of ±0.1 mg. A standard analytical balance was used. Analyte weights were determined by transfers using a Teflon®-coated microspatula and are based on actual weight gain of the NMR sample tube. This avoids weighing errors due to spillage or sample adherence to the microspatula. Weight ranges of the analytes, as shown in Table 1, ran from about 28–145 mg aspirin, 16–74 mg phenacetin, and 13–60 mg caffeine. Trioxane standard weights ranged from about 21–55 mg. The samples were dissolved in about 0.5 ml $CDCl_3$, as required for solution. For tablet analysis, accurately weighed portions of tablet and trioxane standard were triturated together with warm $CDCl_3$ (caution: cancer-suspect agent) and the supernatant filtered through a small cotton plug in a Pasteur pipette into the NMR sample tube.

Integrations were generally obtained as the average of three to five runs using a 10 ppm sweep width, 1 min sweep time, and approximately 0.1 mG radio frequency power (the "auto Integrate" mode of the EM-360A). Since the sweep width and time correspond to 10 Hz s^{-1}, delays between integral scans were not utilized [16] and no signs of saturation problems were observed. Integral steps were quite clearly defined with good reproducibility in measured step heights for successive integral steps. The coefficients of variation for the set of integral step heights for each component in each analytical mixture are included in Table 1.

In cases where relatively large amounts of trioxane internal standard were used, the trioxane integral step could go offscale at amplitude settings that provided adequate step heights for the APC constituents. In such cases, the trioxane peak could be scanned at a lower amplitude. On the EM-360A, the 'Coarse' amplitude control provides accurate and reproducible decade steps and can readily be used for this purpose. The spectrometer employs 1% tolerance precision resistors in the coarse amplitude control. Weighing out smaller amounts of trioxane would eliminate the need for any range change corrections at the cost of greater relative weighing errors. The 'Fine' control has no detents and must be left at a single setting for all the spectral scans of an individual sample. Calculations were based on the integral step height of the aspirin CH_3 (near 2.3δ), the combined integral steps for the two upfield CH_3 singlets of caffeine (near 3.4δ and 3.6δ for the N_1– and N_3–methyls, respectively), and the full integral step for the acetyl CH_3 of phenacetin. This last absorption consists of a major singlet with a small, broad, upfield shoulder. The temperature dependence of the shoulder's appearance led Hollis [1] to attribute this to two rotamers of phenacetin, reflecting rotation about the O=C–N amide C–N single bond. Full integration of the main singlet

Table 1
NMR analysis of APC mixtures

Sample	Mixture composition (mg)*			Found by NMR (mg)†‡			Absolute error (%)§		
	Asp.	Phen.	Caf.	Asp.	Phen.	Caf.	Asp.	Phen.	Caf.
1	44.1	—	23.1	44.0 (2.77;5)	—	23.3 (3.13;5)	−0.12	—	+0.65
2	28.3	31.9	13.5	28.7 (5.00;5)	31.6 (4.34;5)	13.6 (6.17;5)	+1.59	−0.79	+0.48
3	32.9	31.2	60.2	32.2 (2.31;5)	31.5 (2.55;5)	59.2 (1.63;5)	−2.05	+0.98	−1.59
4	45.3	25.9	12.4	45.8 (2.18;3)	26.1 (2.84;3)	12.2 (4.52;3)	+1.10	+0.66	−1.61
5	71.0	15.6	14.3	70.3 (1.04)	16.0 (3.44)	14.9 (3.32)	−1.04	+2.23	+4.20
6	40.4	48.1	30.3	41.1 (1.29)	47.8 (1.68)	29.8 (2.80)	+1.79	−0.62	−1.67
7	144.7	64.6	53.9	144.4 (1.50)	65.1 (2.45)	54.9 (2.32)	−0.21	+0.77	+1.86
8	89.9	64.7	46.0	90.2 (1.72)	64.6 (1.98)	45.1 (0.58)	+0.36	−0.15	−2.06
9	88.7	62.3	—	89.2 (1.58)	62.7 (1.74)	—	+0.56	+0.64	—
10	—	74.1	48.6	—	73.9 (1.78)	49.7 (1.76)	—	−0.27	+2.26
11‖	33.4	23.9	4.8	34.3 (1.11)	23.7 (0.66)	4.9 (3.17)	+2.60	−0.77	+2.91

*Note that actual weights were measured to 0.1 mg and were determined by difference, so that they should be considered as accurate to ±0.2 mg [21].
†Rounded off to nearest 0.1 mg; apparent discrepancies in tabulated errors result from this rounding off.
‡The coefficients of variation (%) and numbers of integral scans (if other than four) are given in parentheses.
§Absolute error is expressed here as: 100% × (found mg − actual mg)/actual mg. Thus, a positive value signifies a 'Found' value greater than the 'actual' value. For a discussion of statistical treatments used here, see ref. [22].
‖Tablet; mixture composition values based on LABEL, corrected for excipient and binder. Errors for this sample are not included in the statistical treatment of the method.

and the shoulder account for the 3H acetyl CH_3 intensity of phenacetin. Our use of the combined integral step heights for *two* of the caffeine methyls (N_1–CH_3 and N_3–CH_3) provides a larger integral intensity for more precise measurement than integration of a single NCH_3 (as was done by the authors of ref. [2]).

Analytes and trioxane were used as received from commercial sources without further purification. Nominal purity was 98% for trioxane obtained from Aldrich Chemical Co. U.S.P. grade aspirin, phenacetin and caffeine are 99.5–100.5, 98.0–101.0 and 98.5–101.0%, respectively.

Results and Discussion

The results are presented in Table 1. In all cases, both accuracy and precision appear to be acceptable, and some improvement is seen relative to those reported earlier. Indeed, improved integration accuracy could provide even better results and might be fruitfully applied here using the technique of an external digital voltmeter, as reported by Johnson and Shoolery [17] and discussed by Waters [18]. Since the trioxane singlet is well separated from neighboring absorptions, spinning sideband intensity and position is relatively non-critical (see below).

The trioxane has an appreciably higher melting point than piperonal, gives no interferences with the proton absorptions of any of the substances being determined, and is about 30 times cheaper than piperonal per integrated proton (by 1984 prices).

It was explicitly decided to make the correction to the N_1–CH_3 integral intensity of caffeine (near 3.4δ) for the contribution produced by the downfield ^{13}C satellite of methyl in aspirin. This correction has been omitted by other investigators [2]. Clearly, the importance of this correction will depend on the relative amounts of caffeine and aspirin present in a particular sample, with high ratios of aspirin making the correction more important. In any case, as in this present work, by simply using the combined integral intensities of both the N_1– and N_3–methyls of caffeine instead of relying on the N_1–methyl alone was done previously [2], the ^{13}C satellite contribution of aspirin to caffeine becomes only half as important and measurement precision is improved. In practice, the observed integral step heights for the analytes over the concentration ranges reported here are such that the ^{13}C satellite contribution is typically of the same order or less than the small step height measurement errors due to instrument sensitivity, etc.

To elaborate further the question of the ^{13}C satellites of aspirin, $^1J_{CH}$ was measured for the aspirin methyl (as a saturated solution in $CDCl_3$ at 28°) as 129.5 Hz ±0.5 Hz. (Hollis [1] had estimated the value as 120 Hz). This coupling constant value not only fortuitously places the downfield satellite on the N_1–CH_3 of caffeine but also puts the upfield satellite on the upfield branch of the phenacetin triplet. However, the enhancement of the phenacetin methyl triplet upfield branch which was observed in some APC samples far exceeded that predicted solely from a ^{13}C satellite contribution from methyl in aspirin. It therefore seems distinctly preferable to avoid use of the phenacetin triplet for calculations.

$^1J_{CH}$ was also measured for trioxane as 167 Hz ± 1 Hz to confirm that the internal standard ^{13}C satellites would not interfere with our analyte peaks. The measured ^{13}CH coupling constants for the aspirin methyl and the trioxane methylene in the present work are consistent with reported values of 130 Hz for acetic acid and 161 Hz for diethoxymethane [19]. Although Hollis had estimated similar ^{13}CH coupling constants (of around 120 Hz) for both the aspirin CH_3 and the caffeine methyl (near 3.4 ppm), the coupling constants were found to be significantly different in the present work. Caffeine displayed ^{13}CH coupling constants for all three methyls of 142 ± 1 Hz ($CDCl_3$ solution, 28°C). This means that although the satellite of the aspirin methyl contributes to N_1–CH_3 of caffeine near 3.4 ppm, this caffeine methyl does not contribute to the aspirin CH_3. The assumption of Hollis that these proton satellites mutually contributed to each other would have required identical ^{13}CH coupling constants. However, the upfield satellite of N_3–CH_3 of caffeine may be close to overlapping the aspirin CH_3 and would likely be included in the integral of the latter. This coincidence results since the difference in chemical shift between the N_1– and N_3–methyls of caffeine approximate the amount by which the ^{13}CH coupling constants of caffeine methyls exceed the ^{13}CH coupling constant of aspirin methyl. Our observed value for $^1J_{^{13}CH}$ of the caffeine methyls is consistent with the value of 133 Hz given for CH_3NH_2 [19], and, perhaps a better analogy, the value of 139 Hz for the methyl of *N*,*N*-dimethylformamide [20].

The present results indicate a bias in the errors of +0.2% for aspirin, +0.4% for phenacetin and +0.3% for caffeine (based on 'found' minus 'actual' values). Other workers reported consistently low values for aspirin and phenacetin and high values for caffeine in tablets [2]. However, since their results were based on 'claimed' values without independent assays, actual tablet compositions were not available for comparison with the values they found by NMR. Hollis [1] reported results for known APC mixtures with mean errors of about −0.9% for aspirin, −1.2% for phenacetin and −0.1% for caffeine; for APC tablets, 'found' values were high for phenacetin and low for aspirin and caffeine (when compared with LABEL values). Ignoring ^{13}C contributions between aspirin and caffeine should contribute to positive errors (if significant) for both these components which is not totally consistent with the results of ref. [2]. While these results are not readily explained, at least part of the differences noted could result from slightly differing purities of reagent batches or consistent biases in integral step height measurements by different workers. The present somewhat high values may reflect a purity for trioxane of less than 100%, although the calculations were based on a nominal 100% purity.

The weights of each analyte in a sample were calculated as follows:

$$\text{mg aspirin} = \text{mg}_T \times \frac{\text{step height}_A}{\text{step height}_T} \times \frac{6}{3} \times \frac{\text{mg}_A/\text{mmol}}{\text{mg}_T/\text{mmol}}$$

$$\text{mg phenacetin} = \text{mg}_T \times \frac{\text{step height}_P}{\text{step height}_T} \times \frac{6}{3} \times \frac{\text{mg}_P/\text{mmol}}{\text{mg}_T/\text{mmol}}$$

$$\text{mg caffeine} = \text{mg}_T \times \frac{\text{step heights}_C - 0.0055\ \text{step height}_A}{\text{step height}_T} \times \frac{6}{6} \times \frac{\text{mg}_C/\text{mmol}}{\text{mg}_T/\text{mmol}}$$

The subscripts A, P, C and T refer to aspirin, phenacetin, caffeine and trioxane, respectively. Integral step heights used are discussed above. The numerical factors (6/3) or (6/6) refer to relative numbers of protons being integrated in trioxane and the analytes. The last terms are ratios of the molecular weights of the analytes and trioxane. Only the caffeine integrals are corrected for contributions from the ^{13}C satellite of aspirin.

Since the present results were determined for known mixtures over a broad range of compositions, we have presented the coefficients of variation for each sample component based on the integral variations within a set. The means of these coefficients of variation for the eleven tabulated samples were 2.05% for aspirin, 2.35% for phenacetin, 2.94% for caffeine and 1.82% for the trioxane standard. These values are essentially consistent with the spectrometer manufacturer's specification of 2.0% average deviation in total integral reproducibility (of a 5% ethylbenzene sample, five scans). A slightly lower value for trioxane could result from more accurate measurements of larger step heights and use of reduced amplitude settings in measuring the standard's integrals. A slightly higher value for caffeine may result from the measurement of smaller integrals for (usually) the minor component. For each sample mixture, the 'found' results reflect the means value based on the averaged integral step heights (three to five scans). Signs are included in the error tabulations, with a positive sign representing a 'found' value greater than the actual. Absolute errors (%) are presented for comparison with Hollis' results.

Based on the results in Table 1, average deviations from the true values were found to be 1.0% for aspirin, 0.8% for phenacetin and 1.8% for caffeine. Hollis [1] had reported corresponding values of 1.1, 2.2 and 3.2%, respectively. The standard deviations for the present work (based on samples 1–10) were 1.24% for aspirin, 0.95% for phenacetin and 2.19% for caffeine. The use of the internal standard described here should facilitate ^{1}H NMR APC assays, and provide improved accuracy [4].

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