A rapid qNMR protocol for the analysis of triclofos sodium in solution state pharmaceutical dosage formulations

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A quantitative $^{31}$P{${}^1$H} nuclear magnetic resonance (NMR) spectroscopy has been used for quantification of triclofos sodium (TCFS) in syrup formulations. Its hydrolysis product 2,2,2-trichloroethanol (TCE) has been quantified by $^1$H NMR spectroscopy. Standard addition to syrup formulations is used for the quantification of these components. Dimethylsulfone (DMS) and hexamethyl benzene-1,3,5-triyltris(methylene)triphosphonate (HMBTP) have been used as internal standards for quantitative $^1$H NMR and $^{31}$P{${}^1$H} NMR respectively. All NMR experiments are performed on 400 MHz NMR spectrometer equipped with a room temperature broadband observe probe. The method is simple, rapid, and easy to implement. It also shows excellent accuracy (recoveries for TCFS 98.9-101.5% and TCE 98.6 to 99.9%), linearity ($r^2$>0.99), range, precision, accuracy and robustness. Four commercially available formulations have been subjected to the developed method. The average concentration for TCFS is found to be 93±4 mg/mL in the commercially available drug samples.

Keywords: Triclofos sodium, drug, 2,2,2-trichloroethanol, nuclear magnetic resonance, 31P NMR, assay

The sedative, sodium 2,2,2-trichloroethyl hydrogen phosphate is commercially available under the trade name triclofos sodium (TCFS). Its use has dwindled since the advent of benzodiazepines in mid-20th century. Nevertheless, it continues to be used by medical fraternity as a second-line treatment for severe insomnia. It also finds use in sedation of subjects before EEG, CT and MRI examinations as it does not suppress epileptiform discharges. TCFS is a prodrug that hydrolyses to form the active drug 2,2,2-trichloroethanol (TCE). Potency of drugs determines their dose-response relationship. It is therefore essential to have accurate analytical methods for identifying and quantifying TCFS and TCE in dosage formulations. The method reported in British Pharmacopeia relies on complete oxidation of a known quantity of the formulation followed by quantitative estimation of total chloride and phosphate species; it does not yield quantitative information about the actual concentration of TCFS and TCE in the formulation.

Hence, there was a need for developing an analytical method for the identification and quantification of intact as well as hydrolyzed triclofos in the pharmaceutical dosage forms. NMR spectroscopy is a primary ratio method of measurement that finds wide application in pharmaceutical analyses. It is a soft technique that requires minimal sample treatment (only dissolution in deuterated solvents and addition of internal standards). The nucleus specific observation allows specific detection, identification and quantification of the components present in complex mixtures. It has the highest linear dynamic range among all the available analytical techniques. Diffusion ordered NMR spectroscopy and hyphenated LC-NMR spectroscopy has been used for analyses of complex mixtures. But these techniques have their own drawbacks.

Therefore, the aim of this study was to develop an operationally simple, robust, quantitative and a reproducible method of identification followed by quantification of TCFS and TCE in its syrup dosage formulations. The $^1$H, $^{31}$P, $^{31}$P{${}^1$H} and $^1$H-$^{31}$P FAST HMQC-NMR spectroscopy has been used for this purpose. Applicability of the developed method was checked on commercially available syrup formulations.

Experimental Section

Materials

Deuterium oxide (minimum deuteration 99.96%), 3-(trimethylsilyl)propionic-2,2,3,3-$d_4$ acid sodium salt (TSP, minimum deuteration 99.8%), methanol-$d_4$ (minimum deuteration 99.8%), internal standard dimethyl sulfone (>99.99%) and TCE (>99% pure) were obtained from Aldrich (Milwaukee, USA). The internal standards, TCFS and hexamethyl benzene-
were synthesised in-house as per reported procedures. They were re-crystallized and dried under high vacuum until their purities were >99.99% (ascertained by $^{31}$P, $^1$H-$^{31}$P FAST HMQC, quantitative $^1$H, $^{31}$P$^{'1}$H) NMR and LC-UV experiments). Water deionised by Milli-Q water purification system (Millipore, USA) was used for preparing stock and working standard solutions.

**Instrumental conditions**

A Bruker Avance-II NMR spectrometer (Bruker Biospin, Switzerland) operating at 400.13 MHz for $^1$H and 161.99 MHz for $^{31}$P was used for the experiments. A 5 mm multinuclear inverse probe-head was used for the Fast-HMQC and $^1$H NMR experiments. Whereas, a 5 mm multinuclear automatically tunable observe probe-head with actively shielded z-gradient coils were used for $^{31}$P$^{'1}$H NMR experiments. The instrument control, data acquisition and data processing were performed by Topspin 1.3 software. All the samples were locked on methanol-$d_4$ and shimmed individually (line-width of DMS signal <0.6 Hz and HMBTP signal < 1.2 Hz), at a pre-calibrated probe temperature of 25±1°C. The relaxation time $T_1$ was determined for the protons and phosphorus nuclei of interest (Table I). The $^{31}$P$^{'1}$H-$T_1$ measurements were performed by using a modified Bruker inversion-recovery pulse sequence “T1ir” incorporating inverse gated$^1$H decoupling by WALTZ16 composite pulse train. The quantitative$^1$H and $^{31}$P$^{'1}$H NMR spectra were acquired using Bruker pulse programs zg30 and zg0ig respectively. The carrier frequency was set at the centre of the region of interest.

**System suitability**

The field frequency locked hump test sample (0.5% CHCl$_3$ in acetone-$d_6$, Bruker Biospin GmbH, Rheinstetten, Germany) magnet was used for shimming the magnet. A Lorentzian lineshape with a half-height linewidth (<0.8 Hz), was obtained for the chloroform signal. This parameter was measured periodically to ascertain suitability of the NMR spectrometer.

**Selection of internal standards**

The quantitative NMR experiments were performed on the basis of peak area of the analytes relative to that of the internal standards. Solubility, Table I — Assignments of NMR resonances of analytes and reference standards

<table>
<thead>
<tr>
<th>Nuclei observed</th>
<th>Analytes/standards</th>
<th>NMR chemical shifts (δ), multiplicity</th>
<th>$T_1$ (sec)</th>
<th>No. of nuclei used for quantification</th>
</tr>
</thead>
<tbody>
<tr>
<td>$^{31}$P$^{'1}$H</td>
<td>TCFS</td>
<td>2.19 (± 0.20) singlet</td>
<td>6.099 (±0.5)</td>
<td>1</td>
</tr>
<tr>
<td>$^{1}$H</td>
<td>HMTPB</td>
<td>4.13 (±0.08) singlet</td>
<td>2.305 (±0.8)</td>
<td>2</td>
</tr>
<tr>
<td>$^{31}$P$^{'1}$H</td>
<td>DMS</td>
<td>3.09 (±0.02) singlet</td>
<td>3.084 (±0.7)</td>
<td>6</td>
</tr>
</tbody>
</table>

*Accuracy expressed (as RSD %)
signal resolution, weighability, low-volatility, stability, reactivity with the matrix components and purity were taken into account for selecting the internal-standards. Deuterated water-methanol (80:20) was added as a co-solvent to obtain a homogeneous solution of a known quantity of dimethylsulfone (DMS) for the estimation of TCE. Quantification of TCFS was performed in aqueous solutions using hexamethyl benzene-1,3,5-triyltris(methylene)triphosphonate (HMBTP), as an internal reference. The chemical shifts were referenced to TSP.

Preparation of standard stock, test solutions for NMR analysis

The method was optimized using samples prepared as per the procedure enumerated in Scheme I. Accurately weighted internal standards DMS and HMBTP were added in two separate (2 mL) volumetric flasks. In each flask 500 µL aliquot of the formulations were added. The content of the flasks containing DMS was made up to the mark (using a mixture of CD$_3$OD-D$_2$O, 80:20). Volume of the other flask, containing HMBTP was made up to the mark using D$_2$O. Six different dilutions were also prepared for the standard compounds TCFS and TCE. All solutions were freshly prepared and they were agitated on a vortex shaker before NMR analysis.

Assignment of NMR spectrum of TCFS and TCE

The $^1$H, $^{31}$P{$^1$H}, $^{31}$P, $^1$H-$^{31}$P FAST HMQC NMR experiments were performed on the pure active components, formulations and after standard addition to the formulations. The results were used for assignment of the signals to be used for quantification (Table I, Figure 1, 2 and 3).

The spin-lattice relaxation time of nuclei of interest was determined. The quantitative NMR spectra were acquired in non-spinning mode using 128 transients preceded by 4 dummy scans and an inter-scan delay (of 20 seconds for $^1$H and 80 seconds for $^{31}$P{$^1$H} NMR experiments). Each free induction decay (FID), consisting of 32k data points was further zero-filled to 64k data points. The FIDs were apodized with 0.2 Hz exponential line broadening function before Fourier transformation. Manual two parameter phase correction was used to obtain high quality absorption line shape followed by automatic baseline correction. These peaks of interest were integrated with respect to the internal standard for which an arbitrary constant value was attributed.

Results and Discussion

Optimization of quantitative NMR conditions

The quantitative NMR conditions were first optimized for the simultaneous determination of the

![Scheme I](image)

Scheme I — Sample treatment and NMR analysis performed on the formulations for quantification of the analytes.
Figure 1 — The representative $^1$H NMR spectrum were recorded in D$_2$O (600 µL) (a) triclofos syrup formulation (100 µL) (b) after standard addition of TCE, expansion of the region of interest is shown in the inset.

Figure 2 — A representative $^{31}$P{$^1$H} NMR spectrum of TCFS syrup formulation in D$_2$O.
analytes (Table I). Once the conditions were optimized, NMR data were acquired to take care of loss of resolution for the analytes. The NMR acquisition is advantageous as the data can be acquired without $^1$H NMR signals of the mobile phase which are very intense, creating dynamic range problems. Several solvent suppression techniques were explored for selective suppression of solvent signals. Since the signal for TCE was close to the HDO signal, baseline distortions were observed to effect the signal integration. Therefore, all further experiments were carried out without solvent signal suppression. The NMR peaks were referenced to TSP and the threshold of the plot was adjusted over noise level to highlight the resonances of the analytes in the mixture.

**Specificity and selectivity**

Specificity of the method depends on signal separation of the analytes and the matrix components. It was ascertained that the peaks of interest of the analytes were well resolved and there was no interference from the other components of the mixture or impurities. Further, identity of the analytes were established by $^1$H, $^{13}$C($^1$H), $^{31}$P, $^{31}$P($^1$H) and $^1$H-$^{31}$P FAST HMQC experiments on the standard compounds. The characteristic $^1$H, $^{13}$C($^1$H), $^{31}$P($^1$H) chemical shifts and $^3$J$_{P,H}$ demonstrated specificity and selectivity of the method (Figure 2). In order to obtain good signal lineshapes, the NMR instrumental parameters viz. magnetic field homogeneity, tuning, matching, pulse calibration and bandwidth and strength of the radiofrequency pulses used for solvent suppression were also adjusted. The NMR processing parameters viz. baseline and phase correction parameters and window functions were also chosen appropriately (refer to Experimental Section).

**Limits of detection and quantification**

The lower limit of detection of the analytes is governed by NMR sensitivity of the observed nucleus, multiplicity of peaks, number of nuclei being observed and signal width. Lower limit of detection (LOD) was determined as the lowest concentration of the analyte resulting in the NMR signal with signal to noise (S/N) of 3:1. The lower limit of quantification...
(LOQ) was taken as the concentration below which the S/N was lower than 10:1 (Table II). The LOQ was found to be 5 mg/mL of the formulation.

**Ruggedness**

Method ruggedness was investigated in terms of the variables potentially arising from the instrumental conditions, data acquisition and processing parameters. In an effort to extend this to the present study, different relaxation delays were used for the analysis. Decreasing the pulse repetition delay <5s was found to affect the signal to noise ratio adversely. The effect of probe detuning i.e. the change in pulse length was investigated by observing the S/N ratio of the analyte signals. The change in pulse length by ±2% showed negligible effect on the NMR signal to noise ratio. Of the various processing parameters—window functions, line broadening parameters and baseline correction parameters were tried and the parameters mentioned herein were found to be best suited for the analysis. No significant influence was observed on the $r^2$ values obtained from the calibration curves of NMR experiments. The reference frequency for the experiments was 2000.65 Hz and spectral width of 11 ppm was used for all experiments.

**Accuracy and Precision**

The NMR experimental parameters were carefully optimized in order to obtain good accuracy and precision the method. The peak areas of the analytes were plotted with respect to the concentration and linear regression equations were obtained. Only the calibration points within the linear region curves ($r^2$ was higher than 0.990 and the residuals lower than 30%) were used for quantification of TCFS and TCE.

**Application of the method**

In order to test the method, four samples from four different manufacturers, bearing different batch numbers were purchased from the market (Table III). Method precision and accuracy was evaluated by quantifying the analytes in control samples spiked with TCFS (25, 75, 125 mg/mL) and TCE (0.550, 0.110, 0.220 mg/mL). Three NMR experiments were carried out in triplicate for each concentration level (Figure 4).

Precision was indicated by the intraday and interday (over a period of 10 days) variations of the quantified analytes vis-à-vis quantity of the analytes spiked, and this was found to be less than 1%. Deviation with respect to chemical shifts were found to be between ±0.08 to ±0.13 ppm for the TCE and TCFS respectively, indicating good repeatability.

<table>
<thead>
<tr>
<th>Sample number</th>
<th>Internal standard (mg/mL)</th>
<th>Wt. of TCFS (mg/mL)</th>
<th>Recovery (RSD %), $^3$P{H} NMR Interday (n=3)</th>
<th>Wt. of TCE (mg/mL)</th>
<th>Wt. of internal standard (mg/mL)</th>
<th>Recovery (RSD %), $^1$H NMR Interday (n=3)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>70.95</td>
<td>125</td>
<td>99.4 (2.0)</td>
<td>0.220</td>
<td>0.150</td>
<td>100.5 (0.5)</td>
</tr>
<tr>
<td>2</td>
<td>35.47</td>
<td>125</td>
<td>99.6 (3.0)</td>
<td>0.110</td>
<td>0.100</td>
<td>98.9 (0.6)</td>
</tr>
<tr>
<td>3</td>
<td>17.74</td>
<td>125</td>
<td>99.8 (2.4)</td>
<td>0.055</td>
<td>0.050</td>
<td>99.1 (0.9)</td>
</tr>
<tr>
<td>4</td>
<td>70.95</td>
<td>75</td>
<td>99.6 (2.2)</td>
<td>0.220</td>
<td>0.150</td>
<td>99.2 (0.8)</td>
</tr>
<tr>
<td>5</td>
<td>35.47</td>
<td>75</td>
<td>99.9 (3.2)</td>
<td>0.110</td>
<td>0.100</td>
<td>101.4 (0.3)</td>
</tr>
<tr>
<td>6</td>
<td>17.74</td>
<td>75</td>
<td>99.3 (1.8)</td>
<td>0.055</td>
<td>0.050</td>
<td>100.0 (0.3)</td>
</tr>
<tr>
<td>7</td>
<td>70.95</td>
<td>25</td>
<td>98.6 (3.2)</td>
<td>0.220</td>
<td>0.150</td>
<td>100.4 (1.0)</td>
</tr>
<tr>
<td>8</td>
<td>35.47</td>
<td>25</td>
<td>99.5 (3.9)</td>
<td>0.110</td>
<td>0.100</td>
<td>101.0 (0.5)</td>
</tr>
<tr>
<td>9</td>
<td>17.74</td>
<td>25</td>
<td>99.2 (3.4)</td>
<td>0.055</td>
<td>0.050</td>
<td>101.5 (0.8)</td>
</tr>
</tbody>
</table>

1 Actual concentrations of DMS, taken as internal standard for quantitative $^1$H NMR analysis
2 Actual concentrations of HMTPB, taken as internal standard for quantitative $^3$P{H} NMR analysis.
3 Accuracy expressed as recovery (precision expressed as RSD %)

<table>
<thead>
<tr>
<th>Formulation code</th>
<th>Formulation type</th>
<th>Volume/pk</th>
<th>Labeled strength (mg/mL)</th>
<th>Strength (mg/mL) by NMR method Mean values (RSD %) (n=5)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Syrup</td>
<td>30mL</td>
<td>100.00</td>
<td>95.8 ($\pm$0.12)</td>
</tr>
<tr>
<td>2</td>
<td>Syrup</td>
<td>30mL</td>
<td>100.00</td>
<td>96.2 ($\pm$0.20)</td>
</tr>
<tr>
<td>3</td>
<td>Syrup</td>
<td>30mL</td>
<td>100.00</td>
<td>93.9 ($\pm$0.25)</td>
</tr>
<tr>
<td>4</td>
<td>Syrup</td>
<td>60mL</td>
<td>100.00</td>
<td>97.6 ($\pm$0.22)</td>
</tr>
</tbody>
</table>
Conclusion

The present work describes the development of a quantitative NMR spectroscopic method for quantification of TCFS and TCE in pediatric syrup dosage formulations. Chemical shift and $3J_{P^1H}$ coupling constants have been used to identify them. The quantitative $^1H$ NMR experiments are optimized with concomitant solvent suppression. The baseline separated signals of the analytes in deuterium oxide: methanol 80:20 v/v. The developed method has shown excellent reproducibility with excellent precision, accuracy and ruggedness. Finally, the developed method has been applied for the quantification of these analytes in commercially available dosage formulations purchased from the market. The only drawback, while using the present hardware, is the requirement of reasonable concentration of the analytes. The most significant and exciting potential of the experiments would be to use higher number of scans, low volume NMR tubes and microcryoprobes.

References