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Short Communication

Reichardt's dye and its reactions with the alkylating agents 4-chloro-1-butanol, ethyl methanesulfonate, 1-bromobutane and Fast Red B – a potentially useful reagent for the detection of genotoxic impurities in pharmaceuticals

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Abstract

Objectives Alkylating agents are potentially genotoxic impurities that may be present in drug products. These impurities occur in pharmaceuticals as by-products from the synthetic steps involved in drug production, as impurities in starting materials or from in-situ reactions that take place in the final drug product. Currently, analysis for genotoxic impurities is typically carried out using either HPLC/MS or GC/MS. These techniques require specialist expertise, have long analysis times and often use sample clean-up procedures. Reichardt's dye is well known for its solvatochromic properties. In this paper the dye's ability to undergo alkylation is reported.

Methods The reaction between Reichardt's dye and alkylating agents such as 4-chloro-1-butanol and ethyl methanesulfonate was monitored spectrophotometrically at 618 nm in acetonitrile and 624 nm in N,N-dimethylformamide.

Key findings Changes in absorption were observed using low levels of alkylating agent (5–10 parts per million). Alkylation of the dye with 4-chloro-1-butanol and ethyl methanesulfonate was confirmed. Reichardt's dye, and its changing UV absorption, was examined in the presence of paracetamol (10 and 100 mg/ml). Whilst the alkylation-induced changes in UV absorption were not as pronounced as with standard solutions, detection of alkylation was still possible.

Conclusions Using standard solutions and in the presence of a drug matrix, Reichardt's dye shows promise as a reagent for detection of low levels of industrially important alkylating agents.

Keywords alkylating agents; genotoxic impurities; pharmaceuticals and Reichardt's dye; UV–vis spectroscopy

Introduction

The need for greater control of genotoxic impurities is a growing area of interest within the pharmaceutical industry, as is the need for rapid and sensitive detection systems.[1] Currently, the detection of genotoxic impurities involves the use of standard analytical chemistry techniques such as HPLC or GC/MS, often in conjunction with sample clean-up measures such as solid phase extraction.

Genotoxic impurities can arise in pharmaceuticals as a result of the synthetic steps involved in drug synthesis, from impurities in starting materials or as a result of by-products from the reactions in a drug's synthetic pathway.[2]

Sulfonic acids are often used to make salts in order to improve drug solubility and absorption properties. The use of sulfonic acids such as methyl, ethyl, benzene and ρ-toluene sulfonic acid can lead to the formation of genotoxic impurities (sulfonic acid esters).[1] GC/MS, HPLC/MS and GC have been used to detect low levels of sulfonic acid esters.[3,4]

Limits for genotoxic impurities in drugs have been formalised in documents such as the position paper published by the Safety Working Party of the European Committee for Proprietary Medicinal Products in 2006,[5] which sets out a number of positions on matters such as threshold mechanisms of genotoxicity and existing methods of analysis. These are well summarised in various reviews.[1,6–9] The main implication for analytical techniques is

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the sensitivity required to meet the limits set forth in the paper based on the allowable daily intake of 1.5 μg/day.[5]

In response to this greater level of regulation, the pharmaceutical industry published its own position paper,[8] which advocates a staged daily intake varying from 1.5 to 120 μg/day depending on the length of time period the medication is taken for. The limits are summarised in Table 1. The lowest detection sensitivity required is for the allowable daily intake (dose) of 1.5 μg/day. For high-dose drugs (e.g. 1g/day), achieving adequate sensitivity is a challenge.

Detection limits of 0.1–0.01 parts per million (ppm) for ρ-toluenesulfonate and benzene sulfonate esters have been found using HPLC/MS.[4] Using GC/selective ion mobility–MS coupled to solid-phase microextraction, sulfonic acid esters were determined to the 5 ppm level in active pharmaceutical ingredients.[3]

Although HPLC and GC/MS methods have been traditionally used for measuring alkylating agents, these methods have limitations of long analysis times, the requirement for sample clean-up in many cases and the need for analysis to be carried out by specialist staff. Additionally, method development and validation activities are made more complex because of the requirement for trace-level analysis. An attractive alternative would be the development of a simple, generic and accurate colorimetric assay based on a dye that changes colour upon alkylation. A potential reagent for such an assay is Reichardt's dye (2,6-diphenyl-4-(2,4,6-triphenyl-1-pyridinio)phenolate; Figure 1).

Reichardt's dye is a well-known solvatochromic dye and has been used to determine the polarity of solvents.[10] It exhibits a strong absorption maximum that changes in response to the solvent conditions. The shifting UV–visible absorption maximum of Reichardt's dye has been used extensively to determine the polarity of solvents.[11] The reduction in absorption of Reichardt's dye has also been used to measure methylation of Reichardt's dye by the alkylating agent iodomethane.[12] Alkylation at the phenoxide moiety causes a reduction in absorption.

Alkylating agents present in pharmaceuticals belong to three main classes: alkyl halides, sulfonate esters and diazonium salts. 4-Chloro-1-butanol is a potential genotoxic impurity caused by the reaction between hydrochloric acid and the solvent tetrahydrofuran; 1-bromobutane is an alkyl halide used in this study to help develop an understanding of the reaction between alkyl halides and Reichardt's dye. Elder and colleagues have published a review on the control and analysis of alkyl and benzyl halides in active pharmaceutical ingredients.[6]

Table 1 Pharmaceuticals Research and Manufacturers of America (PhRMA) Task Force proposal for levels of genotoxic impurities in active pharmaceutical products[8]

Duration of clinical trial exposure (months)	Allowable daily intake (μg/day) for all phases of development
<1	120
1–3	60
3–6	20
6–12	10
>12	1.5

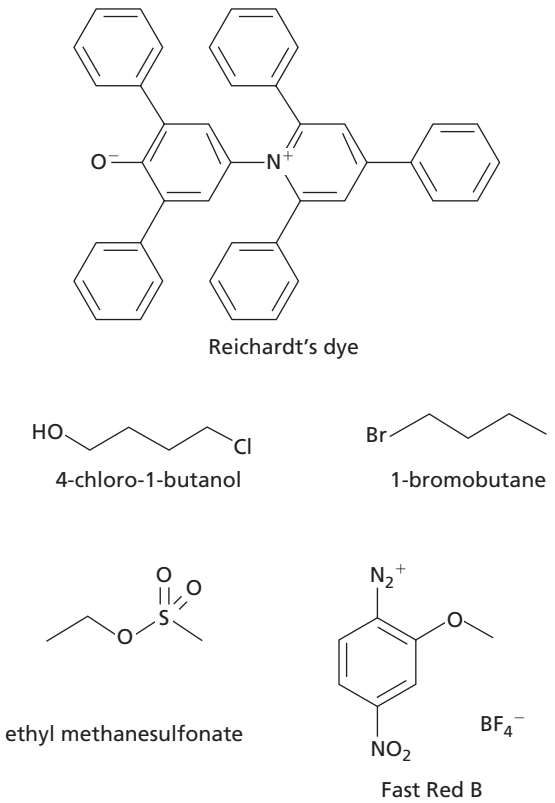

Figure 1 Structures of Reichardt's dye, 4-chloro-1-butanol, 1-bromobutane, ethyl methanesulfonate and Fast Red B.

Ethyl methanesulfonate is a common genotoxic impurity belonging to a class of alkylating agents know as sulfonate esters. The likelihood of producing sulfonate esters is increased if reaction solvent, vessel cleaning solvent or synthetic drug intermediates containing an alcohol group are present during drug synthesis.[1,4] Fast Red B is a diazonium compound representative of a class of compounds used as reaction intermediates during the synthetic stages of drug production when trying to introduce an N–N coupling into a molecule.

The aim of this study was to determine whether an assay system based on Reichardt's dye could be used to measure the genotoxic (alkylating) compounds shown in Figure 1.

Materials and Methods

4-Chloro-1-butanol, 1-bromobutane, ethyl methanesulfonate, diethyl ether, Fast Red B, paracetamol and 2,6-diphenyl-4-(2,4,6-triphenyl-1-pyridinio)phenolate (Reichardt's dye) were purchased from Sigma (Poole, UK). N,N-dimethylformamide (DMF) and acetonitrile were purchased from Fisher Scientific (Loughborough, UK). Deuterated DMF was purchased from Goss Scientific (Great Baddow, Essex, UK).

Stock solutions of 4-chloro-1-butanol, 1-bromobutane, ethyl methanesulfonate and Fast Red B were prepared in DMF or acetonitrile. Subsequent dilutions were then made to

give solutions of the desired concentration for experimentation: 400 μM solutions of Reichardt's dye were prepared in DMF and acetonitrile. One millilitre of dye was mixed with 1.0 ml alkylating compound and then read spectrophotometrically at 618 nm in acetonitrile and 624 nm in DMF. Reactions involving the mixture of 1-bromobutane or ethyl methanesulfonate with Reichardt's dye were heated to 85°C for 4 h and then cooled before reading the absorbance spectrophotometrically. Experiments simulating a drug matrix were carried out in solutions containing paracetamol at a final concentration of 10 or 100 mg/ml. UV–visible spectroscopy was carried out on a UV2100 UV–visible spectrophotometer (Shimadzu, Milton Keynes, UK).

NMR spectroscopy was carried out using a Jeol ECX 400 NMR spectrometer (Jeol, Welwyn Garden City, UK). 4-Chloro-1-butanol and Reichardt's dye were dissolved in approximately equimolar concentrations and then reacted in methanol solvent. The methanol was evaporated and the solid product washed with diethyl ether in order to remove any excess alkylating agent. The solid product was then dissolved in deuterated DMF and investigated by NMR spectroscopy. The reaction between ethyl methanesulfonate and Reichardt's dye was carried out with equimolar amounts in deuterated DMF and monitored by NMR spectroscopy.

Results

Effect of alkylation of Reichardt's dye

Figure 2 shows that the visible absorption for Reichardt's dye decreased with increasing concentration of 4-chloro-1-butanol.

Acetonitrile and DMF were found to be good solvents for this reaction. Figure 3 shows that the decrease in absorption of Reichardt's dye was linear with increasing 4-chloro-1-butanol concentration in both acetonitrile and DMF. DMF produced the larger overall decrease in absorbance, possibly because it is a better solvent for SN2 reactions. For 4-chloro-1-butanol it was possible to establish good sensitivity (10 ppm) and generally good replicate precision was observed.

It was also possible to construct linear response curves for Fast Red B, 1-bromobutane and ethyl methanesulfonate using the changing UV absorption of Reichardt's dye in DMF at 624 nm (Table 2).

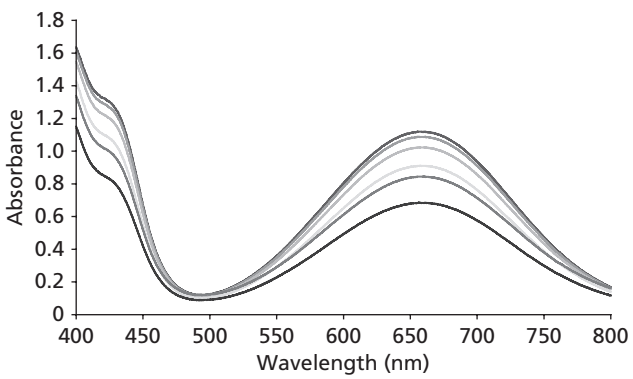

Figure 2 UV–visible spectra for Reichardt's dye. The spectra shown are for Reichardt's dye alone (top line) and with six different concentrations of 4-chloro-1-butanol, in descending order: 8, 20, 40, 60 and 80 μg/ml.

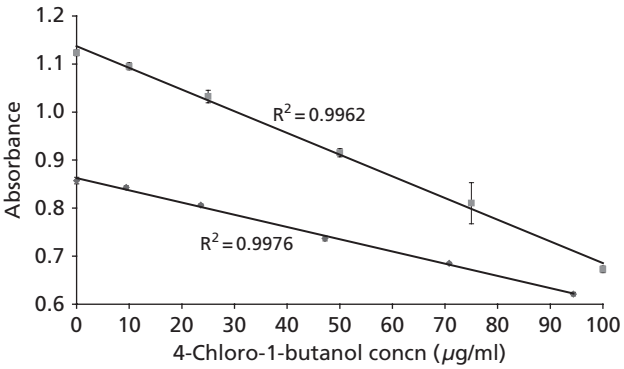

Figure 3 Relationship between 4-chloro-1-butanol concentration and absorption of Reichardt's dye. The UV–visible absorption maximum was read at 624 nm in N,N-dimethylformamide (upper line) and 618 nm in acetonitrile (lower line). Experiments were carried out in triplicate; bars represent SD.

Table 2 Alkylating agents and their associated R^2 values

Alkylating agent	R^2 value
4-chloro-1-butanol	0.9962
ethyl methanesulfonate	0.9756
1-bromobutane	0.9741
Fast Red B	0.9930

4-Chloro-1-butanol and Fast Red B reacted instantly at room temperature with Reichardt's dye. Acceptable linearity was found with 4-chloro-1-butanol, Fast Red B, 1-bromobutane and ethyl methanesulfonate over the range 0–95 μg/ml. High sensitivity was achieved, with absorbance changes detectable at 8–9 μg/ml concentrations.

NMR spectroscopy on the products of the reaction with Reichardt's dye

NMR spectroscopy was carried out on the reactions of 4-chloro-1-butanol and ethyl methanesulfonate with Reichardt's dye. Identification of the reaction products was carried out to verify that the bleaching of dye observed experimentally was due to alkylation and was not due to solvatochromism.

4-Chloro-1-butanol

^{1}H NMR spectroscopy carried out on the product of the reaction between 4-chloro-1-butanol and Reichardt's dye revealed five additional peaks added during the reaction, due to signals from the butyl chain and the OH group of 4-chloro-1-butanol. Integration of the peaks was consistent with addition of the butyl chain to Reichardt's dye. In addition, a dept 135 experiment which can differentiate between signals from CH and CH_3 groups and CH_2 groups was carried out. The experiment confirmed the presence of four CH_2 signals, therefore showing the addition of the butyl chain to Reichardt's dye and thus confirming alkylation by 4-chloro-1-butanol.

Ethyl methanesulfonate

The reaction between Reichardt's dye and ethyl methanesulfonate was slower than the reaction between Reichardt's dye and 4-chloro-1-butanol and could be observed in real time by NMR. Reichardt's dye and ethyl methanesulfonate were dissolved in deuterated DMF and the reaction followed to completion over a 4 h time course by ^{1}H NMR.

NMR spectra acquired during the reaction initially contained strong signals from ethyl methanesulfonate which decreased over the 4 h time course until they could no longer be observed. At the same time, two additional peaks emerged, which were assigned as signals from the alkylated product. Whilst unconfirmed, the reaction mechanism is most probably an SN2 mechanism, with the phenoxide moiety on the dye molecule acting as a nucleophile.[12]

In summary, NMR of the reaction products provided confirmation of alkylation for ethyl methanesulfonate and 4-chloro-1-butanol.

Reactions in the presence of paracetamol

To gain an understanding of how Reichardt's dye behaves in the presence of an interfering drug matrix, it was decided to carry out experiments in the presence of paracetamol at concentrations of 10 and 100 mg/ml.

From Figure 4 it can be seen that the linearity of the response worsened in the presence of 10 and 100 mg/ml paracetamol, with the R^2 value decreasing from 0.9962, to 0.9914 and 0.9937, respectively. The presence of paracetamol reduced the absorbance value at 624 nm, from 1.12 to 0.88 absorbance units at 10 mg/ml, and from 1.12 to 0.28 absorbance units at 100 mg/ml. Scans were run from 800 to 400 nm and the maximum absorption of Reichardt's dye was still found to be at 624 nm. In addition, the decrease in absorbance (gradient of the line) across the 4-chloro-1-butanol concentration range decreased in the presence of paracetamol, particularly at a concentration of 100 mg/ml.

Figure 5 shows a similar effect to Figure 4, with the linearity of the response to ethyl methanesulfonate also worsening in the presence of 100 mg/ml paracetamol. The R^2 decreased from 0.9756 to 0.9600, but linearity was found to be similar between

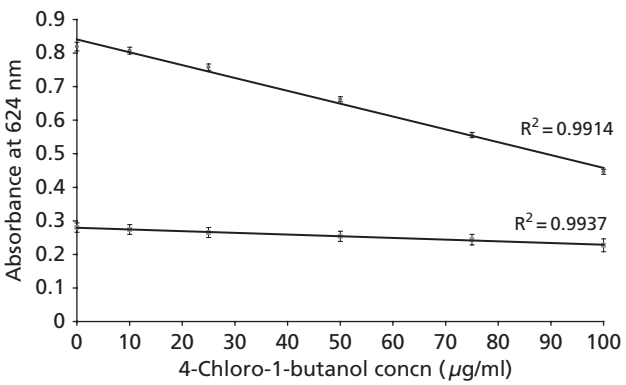

Figure 4 Linear response curve for Reichardt's dye in the presence of 4-chloro-1-butanol and paracetamol. Paracetamol was added at 10 (upper line) and 100 (lower line) mg/ml. Experiments were carried out in triplicate; bars represent SD.

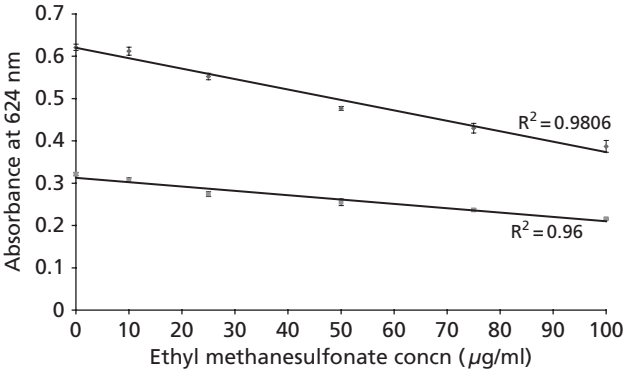

Figure 5 Linear response curve for Reichardt's dye in the presence of ethyl methanesulfonate and paracetamol. Paracetamol was added at 10 (upper line) and 100 (lower line) mg/ml. Experiments were carried out in triplicate; bars represent SD.

standard solutions of ethyl methanesulfonate and solutions containing 10 mg/ml paracetamol (0.9756 vs 0.9806). Again, the presence of paracetamol at 10 and 100 mg/ml caused the absorbance value to decrease similarly to that in Figure 4 (from 1.12 to 0.82 with 10 mg/ml and to 0.46 with 100 mg/ml). In addition, the overall absorbance of the assay decreased in a similar fashion to that in Figure 4.

Results with paracetamol present alongside Reichardt's dye and either 4-chloro-1-butanol or ethyl methanesulfonate show that the presence of drug matrix reduced the efficacy of the assay. Linearity was generally decreased in the presence of paracetamol, and the effect was much stronger with 100 mg/ml paracetamol. The decreases in absorbance with different concentrations of alkylating agent means that differentiation of data points was not equivalent to standard solutions of alkylating agent.

Discussion

Reichardt's dye has been shown to bleach, by alkylation, in the presence of alkylating compounds at concentration levels relevant to genotoxic impurity analysis. The same absorption phenomenon observed for iodomethane[12] was observed for all four of the compounds tested (i.e. decreasing absorption upon reaction). It is therefore appropriate to assume that alkylation of Reichardt's dye has occurred. The dye's loss of colour can be measured by visible spectrophotometry. Bleaching by 4-chloro-1-butanol and ethyl methanesulfonate has been demonstrated by NMR spectroscopy to be the result of alkylation.

In the presence of paracetamol at 10 and 100 mg/ml, the assay lost some of its resolution. This does not invalidate the work however, because there is potential to deal with some of the problems caused by interference by the drug matrix. Controlling apparent pH or buffering the assay may negate some of the interference effects caused by active pharmaceutical ingredients.

Reichardt's dye is highly sensitive to its solvational environment; retaining control of this may therefore help to stabilise the assay. Should it not prove possible to maintain the assay's resolution in the presence of interfering drug matrix, it may be necessary to couple analysis by Reichardt's dye with a sample preparation step. Further activities will

involve testing with other drug matrices, looking at apparent pH or buffering, optimising sensitivity, and the development of analytical sensor technology that incorporates Reichardt's dye for the accurate measurement of alkylating agents in pharmaceutical formulations.

Conclusions

This work has shown the possibility of developing a colorimetric assay for alkylating compounds (e.g. trace impurities in pharmaceuticals). The ability to quickly and simply test for the presence of genotoxic impurities would be of significant benefit. As regulatory scrutiny increases, pharmaceutical companies will be required to demonstrate ever more effective control of genotoxic impurities; a sensitive and rapid assay system is therefore desirable.

Declarations

Conflict of interest

The Author(s) declare(s) that they have no conflicts of interest to disclose.

Funding

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