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Short communication

Trifluoromethanesulfonic acid as a catalyst for the formation of dansylhydrazone derivatives

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Abstract

Trifluoromethanesulfonic acid (TFMSA) is presented as a new, efficient catalyst in the pre-column fluorescent derivatization of the 3-ketosteroid budesonide with dansylhydrazine and compared to the commonly used catalyst trifluoroacetic acid. With TFMSA the derivatization reaction may be carried out at room temperature, with a considerably higher reaction rate compared to previously used acids. The chromatograms also show that TFMSA results in less formation of spurious peaks from the reagent. Derivatization of steroid standard solutions ranging from 0.5 to 64 μM could be performed using identical reaction conditions.

Keywords: Derivatization, LC; Trifluoromethanesulfonic acid; Steroids; Budesonide; Dansylhydrazones

1. Introduction

Derivatization of the ketone functional group for enhanced detection in liquid chromatography is preferentially carried out by hydrazone formation, using a hydrazine label under acidic conditions [1]. An important analyte group amenable to this detection enhancement is the ketosteroids, for which various compounds can be used as labelling reagents, enabling the use of detection principles such as fluorescence [2–4], chemiluminescence [5,6], UV-absorption [7], and electrochemistry [8]. A fluorescent hydrazine frequently encountered as labelling reagent for ketosteroids is dansylhydrazine (DNSH). Since it has been found that different acids influence both the rate and yield of the hydrazone formation, several acids such as hydrochloric acid [9], acetic acid [10], and trichloroacetic acid [11] have been studied as catalysts. Although these acids are capable of catalyzing the reaction, they are not very efficient at room temperature. The most efficient acid known to date appears to be trifluoroacetic acid (TFA), which Koziol et al. [6] found to double the reaction rate compared to trichloroacetic acid (TCA), when used as a catalyst for the dansylation of 3-ketosteroids in an evaporative derivatization procedure.

In addition to enhancing the kinetics of the reaction, the catalyst used for 3-ketosteroid labelling should also be selective in the reaction it promotes. Budesonide, the model compound used in this study, carries two carbonyl groups, in the 3- and 20-positions, and furthermore consists of two epimers with

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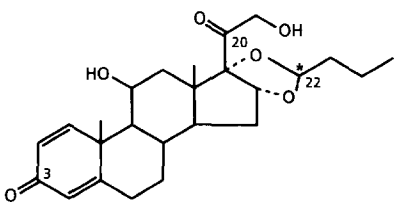

Budesonide

Fig. 1. Structure of budesonide. The star indicates the site of the 22 *R,S* centre.

configurations 22 *R* and 22 *S* (indicated in Fig. 1). Both mono- and difunctionalization may thus take place in the derivatization reaction, and since both *syn*- and *anti*-bonding of the hydrazones is possible, at least four reaction products may thus theoretically be formed from the two epimers of budesonide. It is therefore important to ascertain that a group-specific reaction takes place, and our intention was to establish the conditions required for hydrazone formation at the 3-keto position only. Reich et al. [12] have investigated the reaction between different ketosteroids and 2,4-dinitrophenylhydrazine, and found that the 3-keto group was more reactive than the keto groups in 17- and 20-positions. Weinberger [2] has shown that it is possible to perform a selective derivatization at the 3-position, leaving an unreacted 20-keto group, merely by carrying out the reaction at room temperature, instead of at elevated temperatures.

Trifluoromethanesulfonic acid (TFMSA) is among the strongest known monoprotic Brönsted acids [13]. The acid and its conjugate base possess several important properties, such as extreme thermal stability and a high resistance towards both reductive and oxidative cleavage [13]. Perfluoroalkylsulfonic acids are also much stronger than other inorganic acids, such as sulfuric acid and hydrochloric acid. Although these properties suggest that TFMSA should be an efficient catalyst, it appears not yet to have been used in analytical derivatization reactions. We have therefore investigated the dansylation of the 3-ketosteroid budesonide at room temperature using TFMSA as catalyst. This paper presents the first results from this work.

2. Experimental

2.1. Reagents and solutions

The structure of the steroid used in this study is shown in Fig. 1. Budesonide was obtained from Astra Draco (Lund, Sweden). Dansylhydrazine (5-[dimethylamino]-1-naphthalenesulfonic hydrazide; 98%), trifluoroacetic acid (99%) and trifluoromethanesulfonic acid (98%) were obtained from Aldrich (Steinheim, Germany). Disodium hydrogenphosphate (anhydrous, p.a.) and potassium dihydrogenphosphate (anhydrous, p.a.) were obtained from Merck (Darmstadt, Germany), while acetonitrile (HPLC, Ultragrade) and methanol (HPLC-grade) were obtained from J.T. Baker (Deventer, Netherlands). All water used was purified by Super–Q (Millipore, Bedford, MA, USA) equipment and had an electrolytic conductivity less than 60 nS cm^{-1}. Standard solutions of dansylhydrazine were prepared daily and stored in glass vials at room temperature, protected from light with aluminium foil. Solutions of budesonide, trifluoromethanesulfonic acid, trifluoroacetic acid and dansylhydrazine were prepared in methanol. The mobile phases used for enrichment and separation were based on mixtures between acetonitrile and a phosphate buffer (7.7 m*M* phosphate, pH 7). The mobile phase used for the enrichment contained acetonitrile–phosphate buffer (6:94, v/v), whereas the mobile phase used for separation of budesonide derivatives contained acetonitrile–phosphate buffer (65:35, v/v). Mobile phases were filtered through a 0.47 μm PVDF-filter (Millipore) and degassed for 15 min with He prior to use.

2.2. Derivatization of budesonide with dansylhydrazine

Derivatization was performed in 2 ml glass vials (Chromacol, London, UK) with the reaction mixture protected from light. The budesonide solutions (250 μl) were initially mixed with acid (625 μl), followed by an immediate addition of DNSH (625 μl). The steroid and DNSH concentrations differed in various experiments, and the concentrations are given in the legends to each figure. The mixture was

then allowed to stand on an orbital shaker (IKA Janke and Kunkel, Staufen, Germany) for various periods of time prior to injection on the chromatographic system. Blanks were prepared in a similar way as the samples, but contained only methanol instead of steroid solution.

2.3. Warning

TFMSA is a hazardous reagent, especially when used in alcoholic solutions, as it forms strongly alkylating alkyl trifluoromethanesulfonates (triflates) [13]. Reagents, as well as derivatized samples should therefore be handled with due care. To deactivate methanolic triflate solutions, add an equal amount of 2 *M* aqueous ammonia and leave the solution for an hour or more.

2.4. Chromatographic system

The configuration of the coupled column chromatography system is shown in Fig. 2. It consisted of two blocks, one for enrichment and the other for the separation.

The enrichment block comprised an LC-pump (*Pump 1*; Model 302; Gilson, Villiers le Bel, France), equipped with a Gilson 802 manometric module. The flow-rate was set to 1 ml/min. Derivatized solutions were injected (100 μl) by an LKB 2153 autoinjector (Pharmacia, Uppsala, Sweden) into the enrichment column (*Column 1*, C_{18} 5 μm, 10 mm long by 4.6 mm I.D.; Vydac, Hesperia, CA, USA), which was mounted in the loop position of an LC injector

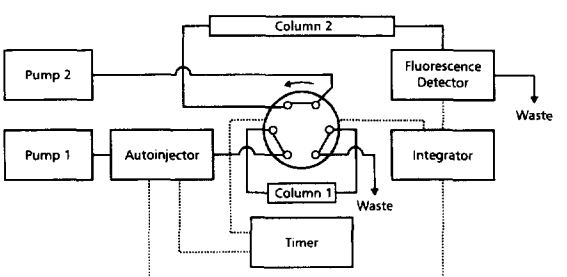

Fig. 2. Schematic drawing showing the configuration of the coupled column chromatography system used for evaluating derivatization yields. See Section 2 for details.

(model 7000, Rheodyne, Cotati, CA, USA) equipped with a Rheodyne 5701 pneumatic actuator. A Crouzet Top 948 counter (ELFA, Stockholm, Sweden) was used as a controller for timing the collection and quantitative transfer of injected solution from *Column 1* into the separation column. A dwell time of 100 s was used before the pre-column was connected into the separation flow system.

The separation block consisted of a CMA Model 250 inert LC-pump (*Pump 2*; CMA/Microdialysis, Stockholm, Sweden) set to a flow-rate of 1 ml/min, and a separation column (*Column 2*, Nucleosil C_{18} 5 μm, 150 mm×4.6 mm I.D.; Skandinaviska Genetec, Kungsbacka, Sweden).

Detection was carried out with a Merck Hitachi F1000 fluorescence detector (Darmstadt, Germany) equipped with a 12 μl flow cell. The excitation and emission wavelengths used were 350 nm and 520 nm, respectively. The detector output was recorded with an electronic integrator (Model 3395; Hewlett Packard, Palo Alto, CA, USA).

3. Results and discussion

Derivatizations of budesonide with TFMSA or TFA as catalysts both led to occurrence of two derivative peaks in the octadecylsilica column/acetonitrile–phosphate buffer (65:35) mobile phase chromatograms. The peaks are marked by arrows in Fig. 3a and Fig. 3b. No peaks were seen eluting later, indicating the absence of difunctionalization of the steroids. The area ratio between the first eluted peak and the late eluted peak remained essentially constant at 2:1 for repeated derivatizations using different reaction times. If difunctionalization took place, we would expect this ratio to vary with reaction time. The 20-keto group is also considerably more sterically hindered than the keto group in the 3-position. We hence conclude that only monofunctionalization in the 3-keto position takes place. The twin peaks can be explained in view of the results of Imai [14,15], who suggested that derivatives resulting from syn- and anti-bonding at the 3-position could be separated on an octadecylsilica column.

When TFMSA and TFA were used for the derivatization of high concentrations of budesonide

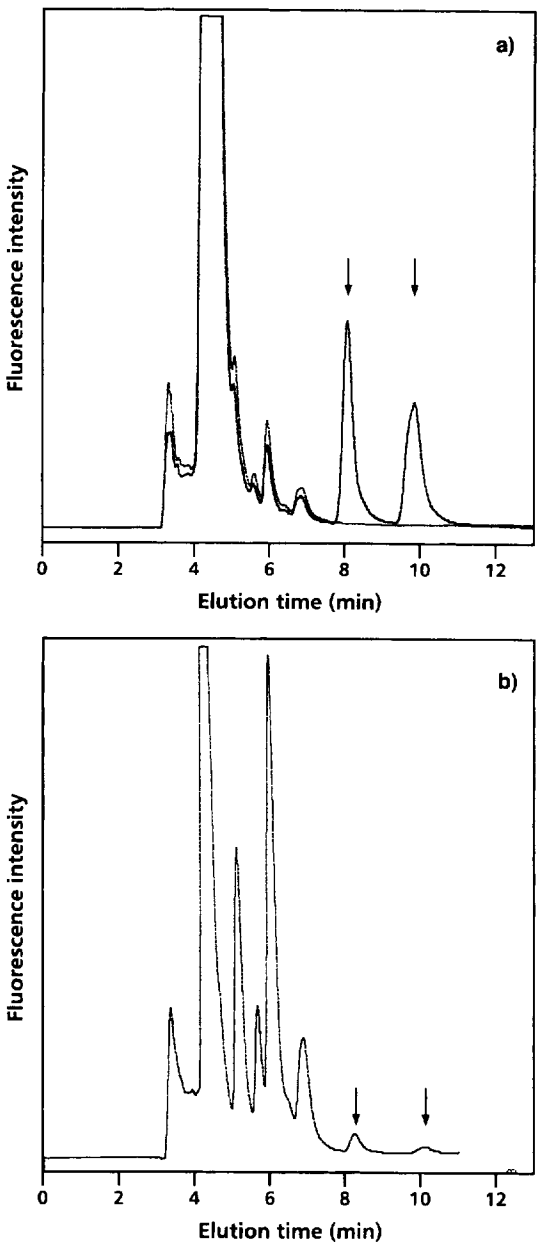

Fig. 3. (a) Chromatogram of a 80 μM standard solution of budesonide derivatized for 100 min using 160 μM dansylhydrazine and 9% TFMSA, superimposed on the blank chromatogram. Arrows indicate the derivatives formed. (b) Chromatogram of a 80 μM standard solution of budesonide derivatized for 100 min using 160 μM dansylhydrazine, and 9% TFA. Arrows indicate the derivatives formed.

under identical conditions, the reaction rate for the TFMSA-catalysed reaction was found to be significantly higher, leading to 95% of the maximum yield in less than 4 h at room temperature. The yield using TFA after the same reaction time was less than 10%; cf. Fig. 3. An illustrative plot showing the yields as function of reaction time could not be included here, and is instead made available as supplemental material on the World Wide Web [16].

Another feature of TFMSA as catalyst was the relative absence of induced peaks in the blank chromatograms. When blank solutions, i.e., methanol without any steroid, were run through the derivatization procedure and separated, no extra peaks appeared in the TFMSA-catalyzed chromatograms (Fig. 4b), whereas a series of spurious peaks, whose magnitude increased with derivatization time, ap-

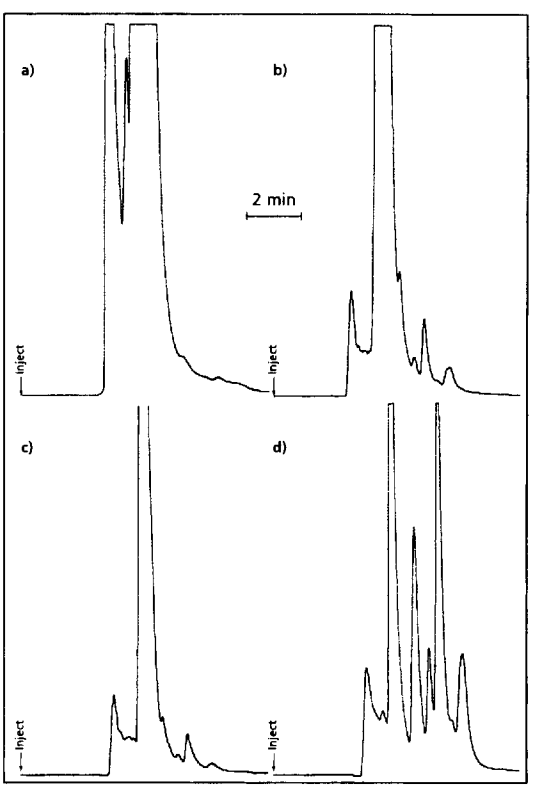

Fig. 4. Chromatograms showing the difference in blank pattern with derivatization time for the investigated catalysts using 160 μM dansylhydrazine, (a) without catalyst, reaction time 300 min; (b) 9% TFMSA, reaction time 300 min; (c) 9% TFA, reaction time 5 min and (d) 9% TFA, reaction time 300 min.

peared with TFA as catalyst; see Fig. 4c and 4d. This indicates that the TFMSA-catalyzed reaction is not only faster, but also produces less reagent by-products than with TFA catalysis.

Using the provisional conditions, five budesonide standard solutions ranging from 0.5 to 64 μM (0.12 to 16 nmol in the reaction mixture) were derivatized with TFMSA as catalyst. A reaction time of 12 h was used in the experiment, conceivably giving a complete reaction even at low steroid concentrations. The linear regression equation for the calibration curve of the budesonide derivatives was described by a slope of 1181 ± 31 V M^{-1} and the intercept 0.007 ± 1 V ($n=9$, 95% confidence interval, $r^2=0.9991$). It is worth noting that data were not obtained by dilution of derivatized high concentration standards, which is a misleading approach often seen in the literature. The reproducibility for nine replicated derivatizations of 1–10 μM standards was 2.5% relative standard deviation (R.S.D.) based on peak height measurements. The limit of detection for budesonide was estimated from calibration curve data to be 1.5 pmol (100 μl injected), based on three times the standard deviation of a low concentration standard solution. The coupled column system was capable of a retention time repeatability of $<0.2\%$ R.S.D. ($n=9$) for the injected steroid hydrazones.

In this work we have shown that TFMSA has the potential of being a highly efficient and clean catalyst for room temperature derivatizations involving hydrazone formation. Our forthcoming studies on TFMSA catalysis will focus on the fundamental parameters, such as temperature, reaction time, and on molar ratios in the reaction, in order to establish optimal conditions for derivatizations at sub-picomole levels.

Acknowledgments

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