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Short Communication

Chemical reduction of FD&C Yellow No. 5 to determine combined benzidine

Valerie M. Davis*

Division of Toxicological Studies, US Food and Drug Administration, Washington, DC 20204 (USA)

John E. Bailey, Jr.

Division of Colors and Cosmetics, US Food and Drug Administration, Washington, DC 20204 (USA)

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ABSTRACT

Data are presented suggesting the presence of the aromatic amine benxidine as a combined impurity in the regulated color additive FD&C Yellow No. 5. The benxidine exists as an azo-dye constituent that forms from free benxidine impurities introduced during the manufacture of the color additive. The presence of combined benxidine is ascertained by sodium dithionite reduction of the azo bonds in the commercial color additive. The resulting reduction products are extracted with chloroform, and the liberated benxidine is determined by high-performance liquid chromatography (HPLC). The levels of benzidine determined by HPLC exceed those levels of benxidine accounted for by direct determination of free aromatic amines in the unreduced color.

INTRODUCTION

FD&C Yellow No. 5 is a color additive approved for use in the United States in foods, drugs and cosmetics, and is subject to batch certification by the US Food and Drug Administration [1]. Regulations limiting the levels of chemical impurities allowed in this color additive were revised. The revised specifications restrict the allowed levels for several aromatic amines, including a limit of 1 ppb (w/w) for benzidine as the free amine [2].

Benzidine that occurs as a contaminant in FD&C Yellow No. 5 probably originates as an impurity in the sulfanilic acid intermediate used to manufacture the color additive [3]. Any benzidine present in the sulfanilic acid starting material may also be diazotized and coupled during manufacture, leading to potential contamination of the color additive by combined benzidine in the form of subsidiary dyes (Fig. 1). When azo dyes are ingested, reduction occurs in the intestine, resulting in cleavage of the azo bond and possible liberation of free aromatic amines.

A method [4,5] has been reported for determining residue levels of unsulfonated (free) aromatic amines in color additives, including FD&C Yellow No. 5. In the method, the amines are first extracted from an aqueous, alkaline solution of the color, and then they are diazo-

* Corresponding author.

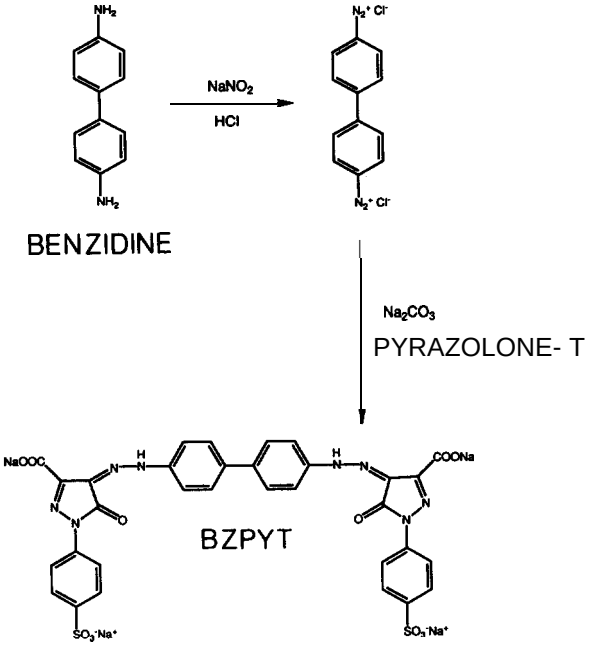

Fig. 1. Diazotization and coupling of benzidine with pyrazolone-T.

tized and coupled. The amines are determined as azo derivatives by high-performance liquid chromatography (HPLC). Although the method determines free amines in the color additive, it does not determine both free and chemically bound amine contaminants.

In this study, the presence of benzidine as a combined impurity in FD&C Yellow No. 5 was investigated. The color additive was chemically reduced at the azo linkage with sodium dithionite. The amines thus liberated were isolated by solvent extraction and determined as previously described [4]. The efficiency of the reduction technique was tested by adding various levels of the combined benzidine impurity to the color additive and measuring the total amount of benzidine released.

EXPERIMENTAL

Materials

Preparation of the diazotization and coupling solutions was described previously [4]. All FD&C Yellow No. 5 dye samples used in this study were from batches of color additive certified during 1985. Extrelut Q.E. disposable columns, Part No. 902050-1, 15 × 4 cm diatomaceous earth, were obtained from EM Science Div., EMS, Inc. (Cherry Hill, NJ, USA). The benzidine-pyrazolone-T coupling product (BZPYT) used as the reference standard (Fig. 1) was synthesized and purified by one of us (J.E.B.) in 1985. The coupling agents, pyrazolone-T and pyrazolone-T acid, were purified by recrystallization twice from hot water; their purity was established by elemental analysis [6]. Water was purified by using the Milli-Q system of Millipore (Bedford, MA, USA). Chloroform and acetonitrile were HPLC grade. Sodium hydrosulfite was purchased from Fisher Scientific, Cat. No. S-309 (Pittsburgh, PA, USA). All other reagents were analytical grade and purchased from commercial sources.

BZPYT standard solutions for calibration

Aqueous stock solutions containing BZPYT reference standard at concentrations of 0.125, 0.25, 0.5, 0.75, 1.0 and 1.25 μg/ml were used to prepare the calibration solutions. For -each calibration solution, 1 g of FD&C Yellow No. 5 determined to be free of benzidine when chemically reduced was dissolved in 30 ml of water, and a l.O-ml aliquot of stock solution was added. The concentrations of BZPYT reference standard in the calibration solutions corresponded to 27, 53, 106, 159, 212 and 265 ppb of free benzidine after liberation of the amine by chemical reduction.

Chemical reduction of FD&C Yellow No. 5

A solution of 1.0 g of FD&C Yellow No. 5 in 30 ml of water was prepared in a round-bottom flask and maintained at 80°C on a hot plate. The pH of the solution was initially adjusted to approximately 8.5 by adding 5 *M* NaOH. Sodium dithionite was slowly added to the solution in small portions until a 10% excess of dithionite over the theoretical amount required for complete reduction had been added (a molar ratio of 2.2 to 1 was employed). This usually required the addition of approximately 0.9 g of dithionite. The sodium dithionite was added in small (mg) quantities until the pH dropped to 7.0. The pH was then readjusted to 8.5 with 5 *M* NaOH.

Nitrogen was bubbled through the solution during the entire reduction procedure to prevent oxidation before the extraction procedure.

Extraction

The extraction step was conducted as described by Richfield-Fratz et *al.* [6] except that the reduced-dye solution (approximately 30 ml) was allowed to cool to room temperature while nitrogen was gently bubbled through the solution. The solution containing the reduced color additive and liberated aromatic amines was carefully poured into the Extrelut cartridge. A few milliliters of water were added to wash the flask, and the liquid was drained into the cartridge. The cartridge was washed with four 25-ml portions of chloroform, and the chloroform eluate was collected in a 250-ml round-bottom flask. A 5-ml portion of 0.05 *M* H_2SO_4 was added to the chloroform extract instead of 5 ml of 0.005 *M* H_2SO_4. Extracts that were not immediately diazotized and coupled were stored in a stoppered round-bottom flask and refrigerated until the following day.

Diazotization and coupling

The diazotization and coupling procedure used was previously reported [5]. A 2-ml portion of 0.1% $NaNO_2$ was added to the flask, the contents were gently swirled to rinse the flask walls and the flask was chilled in an ice bath for 15 min. After 2 ml of the pyrazolone-T coupling solution was added, exactly 13 drops of 0.1 *M* NaOH were added to ensure an alkaline pH during coupling. Each coupled extract was redissolved in 5 ml of water before HPLC determination.

High-performance liquid chromatography

All HPLC determinations were performed within 24 h of extraction, as previously described [5]. A Waters Model 440 dual-channel UV-Vis detector (Waters Chromatography, Division of Millipore, Milford, MA, USA) (2 V output) was set at 436 and at 600 nm to monitor the eluates. A Biosil ODS 5-μm column (25cm x *4.6* mm I.D.) with a 250-μl injection loop was used at ambient temperature for all separations. A 3-ml aliquot of coupled extract was used to flush the loop before each injection of extract.

RESULTS AND DISCUSSION

The retention times of the products obtained from chemical reduction and analysis of a BZPYT reference solution were determined (Fig. 2A). Although benzidine is usually determined (as the benzidine-pyrazolone-T coupling product) at a detector wavelength of 436 nm, other amine coupling products also absorb at this wavelength and may interfere with the determination. However, at 600 nm one major peak appears at 20.6–20.8 min, the retention time of the benzidine-pyrazolone-T coupling product. Thus, the presence of a chromatograph-

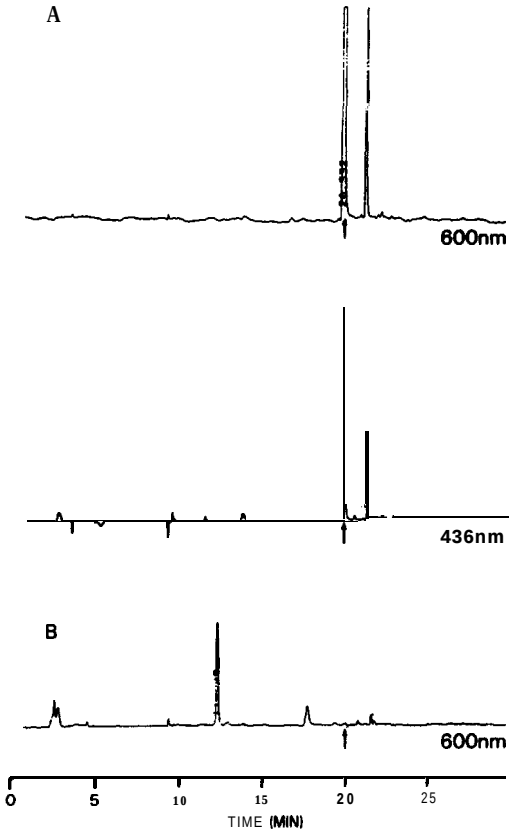

Fig. 2. (A) HPLC chromatograms from analysis of BZPYT reference standard. Detection at 436 and 600 nm. Approximate retention time = 20.7 min. (B) HPLC chromatogram from analysis of FD&C Yellow No. 5 batch containing no chemically bound benzidine or aniline. Detection at 600 nm.

ic peak at both detection wavelengths and calculation of the response ratio (data not shown) provide confirmation of identity during analysis. The small peak appearing just after the major response in the chromatograms in Fig. 2A is probably the monoazo derivative formed when only one benzidine amine group is diazotized.

Fig. 2B shows the HPLC chromatogram obtained from analysis of a previously certified batch of FD&C Yellow No. 5 that was found to be free of both chemically bound benzidine and aniline. (The large amount of aniline that may be produced upon reduction of some commercial batches of FD&C Yellow No. 5 may swamp the HPLC response of the analyte [4,5].) All calibration solutions were chemically reduced and analyzed as described above, and the data were evaluated statistically to measure the linearity of response [7]. The correlation coefficient of 0.97 indicates sufficient linearity over the entire calibration range to allow use of the regression equation to estimate levels of combined benzidine in commercial batches of color, especially at the higher levels.

A total of 14 commercial samples of FD&C Yellow No. 5 were analyzed for total benzidine content by this procedure. The survey included batches submitted for certification during 1985 before restrictions were imposed for aromatic amine residues. The survey also included the batch used in toxicological tests conducted to establish the safety of the color additive. The results of the survey are shown in Table I. The HPLC chromatogram obtained from analysis of the FD&C Yellow No. 5 pharmacology batch (Fig. 3) shows one large peak, at a retention time of approximately 17.5 min, corresponding to aniline, which was liberated during reduction of the color. The chromatogram has only a small peak at the retention time expected for benzidine, suggesting that the test sample does not contain significant levels of combined benzidine.

Fig. 4 shows the HPLC chromatograms obtained for a reduced and for an unreduced commercial batch of FD&C Yellow No. 5 (sample 7 in Table I). Note that the unreduced color produced only a small peak for aniline and no detectable response for benzidine. However, the reduced color produced a large peak for aniline and a small but detectable response at the retention time of the benzidine coupling product. The level of combined benzidine in this batch of color, estimated from the calibration data, is 69 ppb calculated as free benzidine. Similar analysis

TABLE I

HPLC DETERMINATION OF TOTAL BENZIDINE IN COMMERCIAL SAMPLES OF FD&C YELLOW NO. 5.

Sample	Distributor	Retention time (min)	Benzidine found (ppb)
1	A	20.7	245
2	B	20.6	61
3	B	20.7	93
4	C	20.7	40
5	D	20.8	105
6	E	20.7	23
7	F	20.7	69
8	G	20.8	147
9	C	20.7	40
10	H	20.7	19
11	I	20.8	56
12	A	20.6	241
13	F	20.6	38
14	G	20.6	72

[a] Calculated as free benzidine.

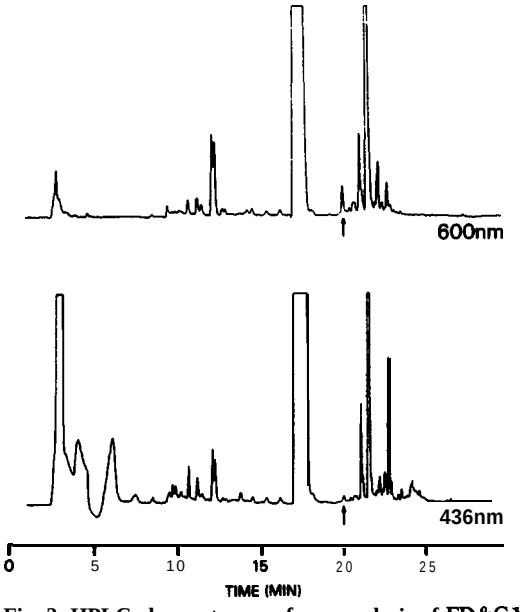

Fig. 3. HPLC chromatogram from analysis of FD&C Yellow No. 5 pharmacology sample. Detection at 436 and 600 nm.

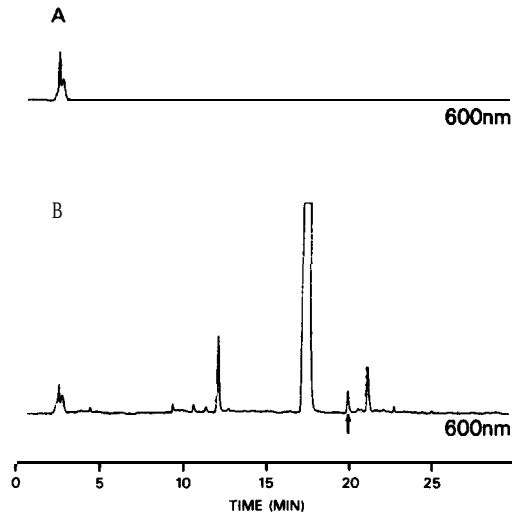

Fig. 4. HPLC chromatograms from analysis of FD&C Yellow No. 5, sample 7: (A) without reduction; (B) with reduction.

of sample 1 in Table I produced the equivalent of 245 ppb of free benzidine, which is the highest level of combined benzidine found in the survey. Analysis of the corresponding unreduced sample found no detectable free benzidine.

The results obtained in this study show that commercial batches of FD&C Yellow No. 5 may contain benzidine as a chemically bound, azo subsidiary color. Although the estimated levels of combined benzidine reported here exceed those observed for free benzidine, the samples surveyed in this study were all manufactured before restrictions were imposed on allowable levels of free aromatic amines. Additional work is under way at the US Food and Drug Administration to refine the analytical methodology used for determining chemically bound benzidine and to develop more complete HPLC characterization of the benzidine analyte. The results presented here will allow an assessment of the effect of restricting the level of free aromatic amine content on the level of total aromatic amine content in the widely used food color.

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