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Sensitive Analysis of Genotoxins by HPLC-ECD Using Boron-Doped Diamond Electrochemical Cell

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ABSTRACT

Genotoxic materials are compounds that have the potential to chemically modify DNA and are considered to be mutagenic and carcinogenic. Furthermore, genetic modification to the DNA contained within an organism's gametes can adversely effect descendants who have never been exposed to the genotoxin. Many genotoxins (for example, intermediates, catalysts, and solvents) can be present during the synthesis of an active pharmaceutical ingredient (API) and, due to their potential toxicity, are being limited to a daily exposure of not more than 1.5 µg.[1,2]

Electrochemical detection (ECD) has been used to determine both the genotoxin (for example, spin traps, TEMPO, and aminopyridines) as well as adducts formed with DNA bases.

In this study, an HPLC-ECD method was developed, with a sensitivity of 200 pg on column for alkyl tosylates and 400 pg on column for alkyl besylates. An isocratic HPLC system and a Coulochem® III electrochemical detector, equipped with a boron-doped diamond (BDD) electrochemical cell, were used for these determinations. Separations were achieved using a perchlorate mobile phase flowing through a fused-core C18 HPLC column.

Calibration curves were determined for methyl, ethyl, and *n*-propyl tosylates, from 200–12,800 pg on column, with linear correlation coefficients > 0.999. Replicate injections were found to have an RSD < 6% for all concentrations, except for the *n*-propyl tosylate at 180 pg on column. Calibration curves were determined for the methyl, ethyl, and *n*-butyl besylates, from 400–12,700 pg on column, with correlation coefficients > 0.999. Replicate injections were found to have an RSD < 6% for all concentrations, except for the *n*-butyl besylate at 800 pg on column.

The data presented here show that HPLC-ECD can measure numerous genotoxins with selectivity and sensitivity. Future work will investigate its applicability to the measurement of genotoxins in drug formulations.

INTRODUCTION

Genotoxic impurities (GTIs) have come under increasing regulatory scrutiny for pharmaceutical products. Exposure limits of no more than 1.5 µg/day are typically required. Available techniques for their quantitation are limited. Two classes of compounds that can be found in synthetically produced APIs include known genotoxic compounds, besylates and tosylates, used as protecting groups during a synthetic process. The analysis of these genotoxic compounds requires significant sensitivity because they often exist at ppm levels compared to the API.

HPLC with electrochemical detection (HPLC-ECD) is extremely sensitive and has been used previously to measure the genotoxic aminopyridines,[3] as well as DNA adducts formed from the interaction of nucleic acids and a genotoxic material.[4,5] ECD, with a BDD working electrode at high potential (> +1800 mV relative to Pd) can be used to detect tosylate or besylate compounds to 190 and 400 pg on column, respectively. The mechanism is believed to involve the formation of hydroxyl free radicals, their attack of the benzene ring, and EC monitoring of the resulting phenol.[6]

The sensitivity and quantitative potential of this method is demonstrated here for six alkylated sulfonate esters, three alkyl besylates, and three alkyl tosylates. During development, it was found that using a degassed mobile phase reduced the response for besylates but not for tosylates. Interestingly, the incorporation of < 1 mM hydrogen peroxide enhanced the signal from besylates and provided a stable baseline. The reason for these findings are still under investigation. Adding hydrogen peroxide provides a stable baseline, and a controllable level of response for both types of analytes.

EXPERIMENTAL

ESA Coulochem III Parameters:

Analytical Cell:	ESA 5040 BDD
Guard Cell:	+1000 mV (vs. Pd reference)
BDD Cell:	+2000 mV for besylates (vs. Pd reference) +1900 mV for tosylates (vs. Pd reference)
Filter:	5 s

HPLC Parameters:

EC-compatible HPLC system, with degasser.

Column:	Halo® C18, 3 x 75 mm, 2.7 µm
ECD Cell and Column Temperature:	35 °C
Sample Temperature:	10 °C
Flow Rate:	0.45 mL/min, isocratic
Injection Volume:	10 µL
Mobile Phase for Tosylates:	40 mM Sodium perchlorate, 20 mM perchloric acid, in water/acetonitrile (65:35)
Mobile Phase for Beslyates:	40 mM Sodium perchlorate, 20 mM perchloric acid, 980 µM hydrogen peroxide in water/acetonitrile (65:35)

Sample Preparation: Standards were dissolved in acetonitrile and dilutions were made with the appropriate mobile phase.

RESULTS AND DISCUSSION

The method for the alkyl tosylates used a degassed mobile phase containing the perchlorate buffer. It was found that the baseline would steadily increase over time. After attributing this to the re-absorption of air into the mobile phase, a degasser was placed into the system. This dramatically lowered the background currents and noise, while increasing the sensitivity of the tosylate response. However, oxygen from the samples was still greater than that in the mobile phase, causing the broad peak seen between 1.5 and 3.0 min in Figure 1. This broad peak does not interfere with the results obtained for the tosylates.

The precision values for three replicate injections are provided in Table 1. The calibration plots are presented in Figure 2. All RSD values were < 6% down to the 192 pg on column amount, with the exception of the *n*-propyl tosylate, due to it being a broader peak, which decreases precision.

For the quantitation of besylates, the conditions used for the analysis of tosylates did not work. Before the degasser was added to the system, both besylates and tosylates responded together. With the presence of the degasser in the HPLC system, the besylate responses vanished. After hydrogen peroxide was added to the degassed mobile phase in small amounts, the response of the besylates, the background current, and noise all increased. However, it was found that just 25 µL of 30% hydrogen peroxide per liter of mobile phase was ideal, providing a sufficient analyte response while maintaining a manageable amount of added background current and noise to the baseline.

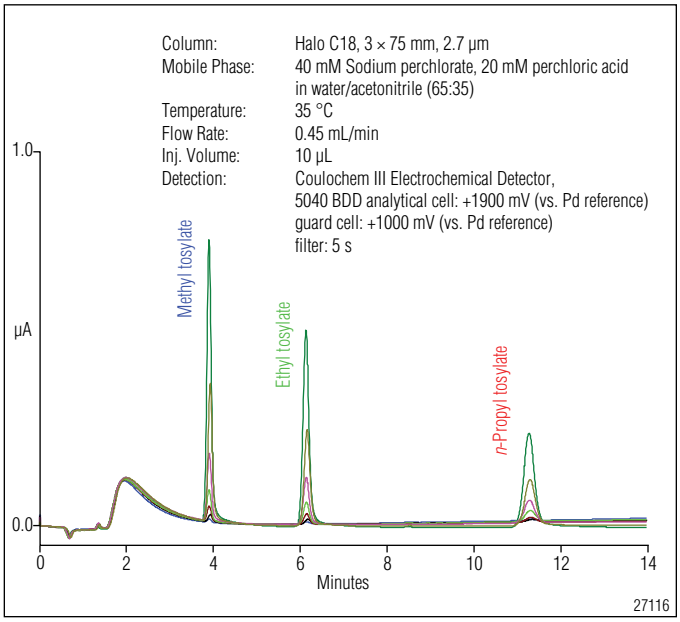

Figure 1. Alkyl tosylates by HPLC-ECD at +1900 mV, relative to palladium, from 192–12,300 pg on column.

Table 1. Precision Results for Alkyl Tosylates by HPLC-ECD, from 192–12,300 pg on Column

Tosylate Amount on Column (pg)			Precision for Tosylates (%RSD, n = 3)		
Methyl-	Ethyl-	*n*-Propyl-	Methyl-	Ethyl-	*n*-Propyl
12300	11700	11500	0.77	1.07	0.33
6150	5850	5750	0.74	0.42	2.39
3075	2925	2875	0.94	0.98	2.31
1538	1463	1438	1.02	0.93	2.73
769	731	719	2.21	0.38	1.72
384	366	359	1.18	4.54	5.08
192	183	180	2.97	5.83	16.18

- All correlations were found to be linear
- All tosylates showed similar response
- All correlation coefficients > 0.999
- Methy and ethyl tosylates are quantifiable down to < 200 pg on column

With the introduction of the hydrogen peroxide, the besylate response also required some time for the electrode response to stabilize. Figure 3 presents sequentially numbered chromatograms taken every 6–12 min, showing the relative increase in response with time. Once the chemistry stabilized, it was possible to analyze standards to below 400 pg on column, as shown in Figure 4, with precision results provided in Table 2.

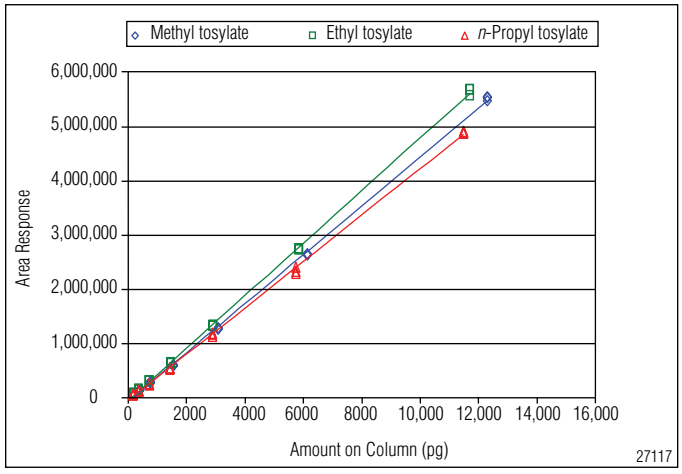

Figure 2. Calibration plots of three alkyl tosylates by HPLC-ECD at +1900 mV, relative to Pd.

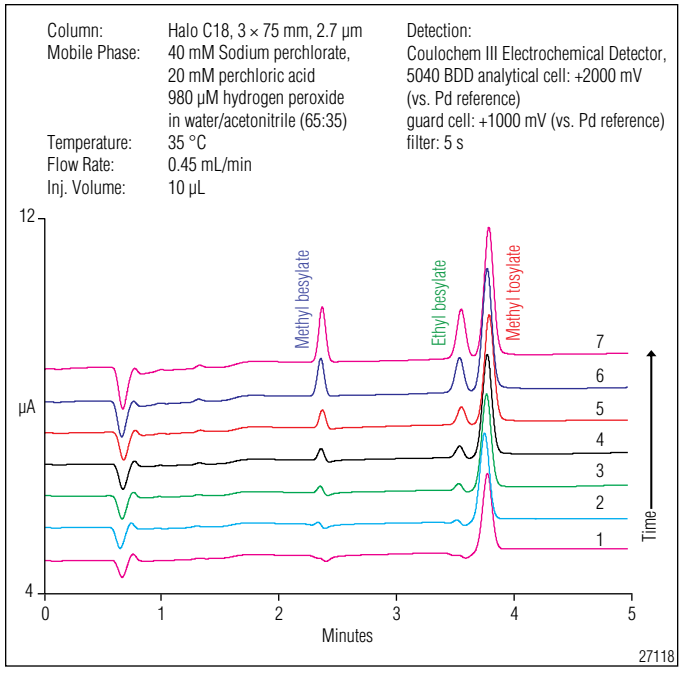

Figure 3. Methyl tosylate and two alkyl besylates response changes over increasing time after addition of H_2O_2.

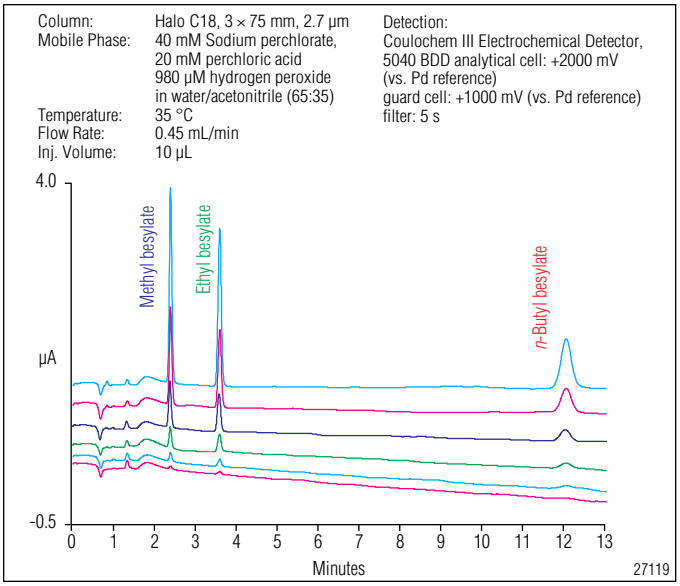

Figure 4. Alkyl besylates by HPLC-ECD at +2000 mV, relative to palladium, from 359–12,700 pg on column.

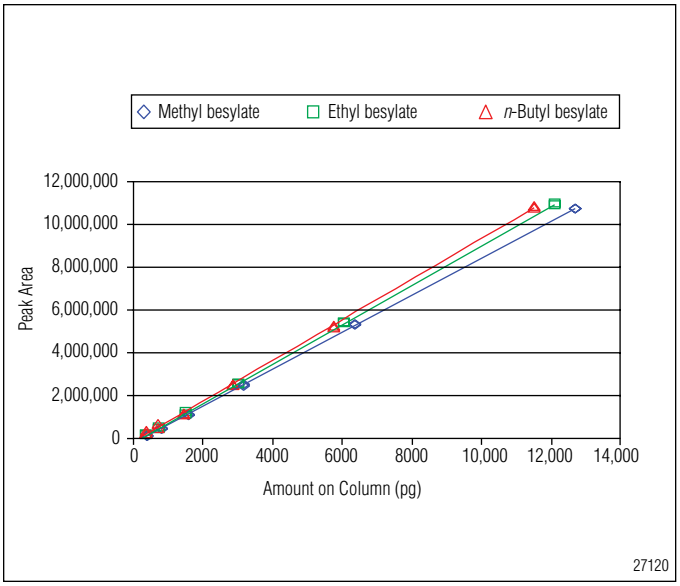

Figure 5. Calibration plots of three alkyl besylates by HPLC-ECD at +2000 mV, relative to Pd.

Table 2. Precision Result for Alkyl Besylates by HPLC-ECD, from 359–12,700 pg on Column					
Besylate Amount on Column (pg)			**Precision for Besylates (%RSD)**		
Methyl-	**Ethyl-**	***n*-Butyl-**	**Methyl-**	**Ethyl-**	***n*-Butyl-**
12700	12100	11500	0.1	0.4	0.6
6350	6050	5750	0.5	0.5	0.8
3175	3025	2875	2.3	1.6	1.5
1588	1513	1438	2.1	6.8	2.9
794	756	719	5.2	9.0	26.3
397	378	359	15.9	14.1	33.0

- All correlations were found to be linear
- All besylates showed similar response
- All correlation coefficients > 0.999
- Methyl and ethyl besylates are detectable down to ~400 pg on column

For API samples, it might be necessary to either extract the sulfonate esters from the sample, or to divert the API from the electrochemical cell so that these minor analytes can be determined without interference from the more concentrated API.

Table 3. LOD and LOQ Values for Sulfonate Esters		
Sulfonate Ester	**LOD (pg on column)**	**LOQ (pg on column)**
Methyl tosylate	50	100*
Ethyl tosylate	50	100*
n-Propyl tosylate	120	360
Methly besylate	270	800
Ethly besylate	410	1230
n-Butyl besylate	460	1370

With these sensitivities, a 250 mg API capsule, taken 4 times a day, would have a limit of 0.375 µg of GTIs/capsule, or 1.5 ppm (µg GTI/g API). Assuming that *n*-butyl besylate was the GTI present, the sample is dissolved, the GTIs extracted, dried, and redissolved in 1 mL of acetonitrile, and then the sample is diluted with one equivalent of aqueous buffer, the amount on column would be 1875 ng, which would be detectable and quantifiable under these conditions.

CONCLUSIONS

A simple, reliable, and sensitive ECD-based method was developed for the measurement of two classes of genotoxic impurities (alkyl tosylates and alkyl besylates) with LODs of < 190 and < 470 pg on column, respectively, for the analytes tested here. The lower potentials used for the analysis of tosylates resulted in better sensitivity for these compounds. The assay has the sensitivity required for routine measurement of these impurities in a pharmaceutical formulation with a linear response over three orders of magnitude.

It was interesting to note the differential effects of degassing and inclusion of hydrogen peroxide in the mobile phase on the electrochemical activity of besylates and tosylates. Although the exact mechanism remains elusive we plan to investigate this phenomenon more fully.

This method presents the basic method that provides low-level detection for these GTIs, with linear response for quantification, which can be adapted for the analysis of alkyl tosylates and alkyl besylates using a BDD analytical cell for HPLC-ECD analysis. The higher LOD values for the larger akyl sulfonate esters are the result of the isocratic elution and the associated peak broadening. This may be addressed through gradient-elution HPLC, which can be performed with the BDD analytical cells.[7]

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