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應用手冊

Lower Drug Metabolite Detection Levels for *In Vitro* and *In Vivo* Samples Using Q-Tof Enhanced Duty Cycles

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Abstract

In this application note, a UPLC-MS/MS method will be described, allowing lower concentration to be detected for metabolite identification.

Introduction

The most challenging step in metabolite identification is the detection of very low level metabolites, particularly with in *vivo* samples. Typically, very little sample preparation is carried out when analyzing these samples in order to keep them as intact as possible, avoiding the risk of polar metabolite loss in an extraction procedure. Urine samples are usually diluted, while for plasma, samples are protein precipitated. As standards are rarely available, analysis occurs with little prior knowledge of expected results. This is particularly evident in drug development, where very low levels of circulating metabolites need to be identified in a sea of endogenous interferences. Thus, searching for low-level putative metabolites in complex matrices is a significant challenge.

The introduction of Waters UltraPerformance LC (UPLC) System has revolutionized this type of analysis through major advances in separation efficiency and sensitivity over traditional HPLC. However, in hyphenated techniques such as this, the quality of the MS detection remains a critical factor in determining low level metabolites, especially as drugdevelopment follows a trend of increased potency and lower dosing levels. This applies not only to full scan MS data, but also places more stringent requirements on MS/MS information quality-vital to determine the structure and site of biotransformation.

In this application note, a UPLC-MS/MS method will be described, allowing lower concentration to be detected for metabolite identification. Complementing superior UPLC chromatographic separations, the Waters Micromass Q-Tof Premier with its enhanced duty cycle (EDC) mode provides enhanced sensitivity, and thus, maximizes the amount of information obtained from a single injection of a complex sample. In this mode, the duty cycle of the mass spectrometer can be increased for specified *m/z* values, providing significant sensitivity enhancements. This is achieved by utilizing novel T-Wave[1] (Traveling Wave) collision cell technology to shape the ion beam into packets of ions. These ion packets are then released from the collision cell at certain intervals with the pusher of the oa-Tof, synchronized to operate as the ion of interest enters the extraction region (Figure 1). EDC can be operated in both MS and MS/MS modes of analysis as well as precursor ion discovery (PID) experiments. The increases in sensitivity offered by EDC are variable, depending on the *m/z* values. Increases of

an order of magnitude or more can be achieved for ions of *m/z* values of 250 and below.

Waters ACQUITY UPLC System with the Waters Micromass Q-Tof Premier Mass Spectrometer

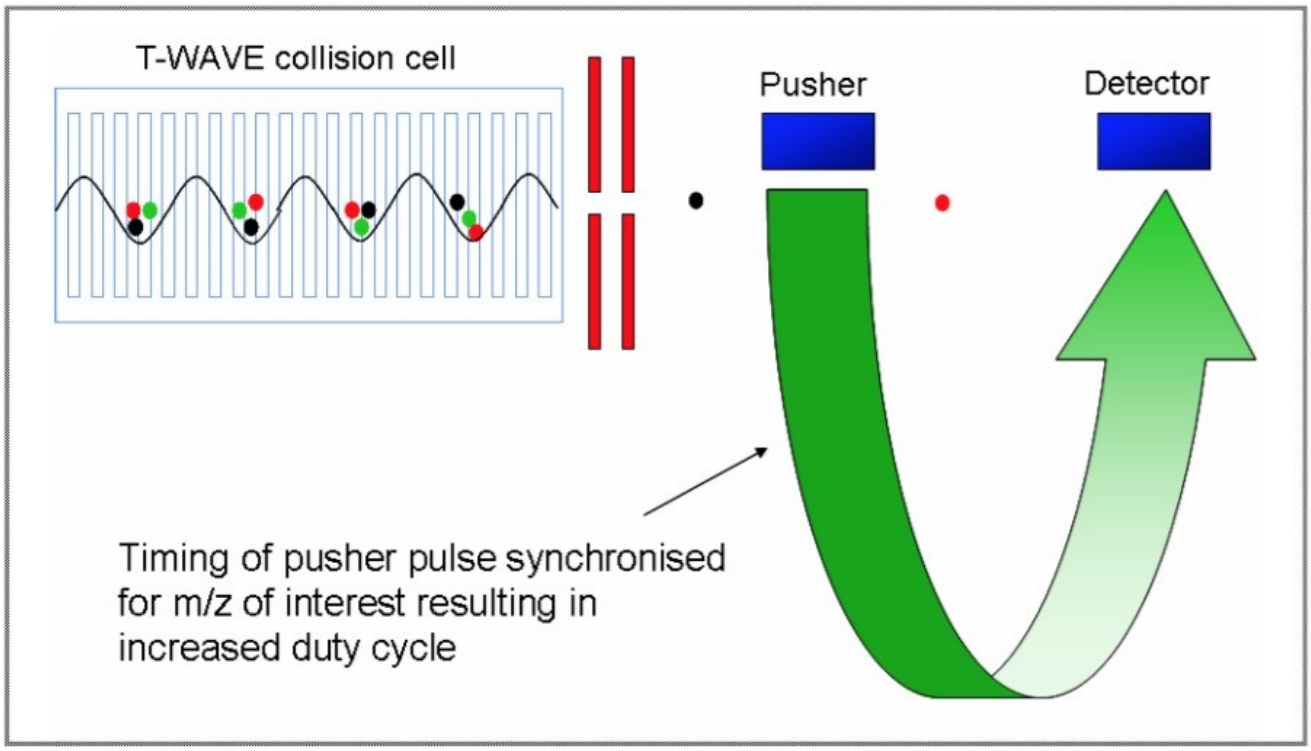

Figure 1. T-Wave collision cell and synchronization with the pusher, allowing specific ions of interest to be detected (in this case, only the “green”ion is synchronized and detected).

Experimental

In this study we will demonstrate the use of the Q-Tof Premier's EDC mode for the analysis of low level metabolites of Indinavir *in vivo* and Propanolol *in vitro*.

Sample Preparation

In vitro metabolism: Propanolol was incubated using rat microsomes at a 1 µM level. The incubation period was 60 minutes at 37 °C, in a solution of 50 mMol potassium phosphate (adjusted to pH 7.4 with NADPH). The reaction was then terminated with 2 volumes of cold acetonitrile to 1 volume of sample. The samples were stored frozen at -80 °C prior to LC-MS analysis.

In vivo metabolism: Lister Hooded rats were orally administered Indinavir at a dose of 30 mg/kg. Urine was collected on dry ice from 0 to 8 hrs. after administration and was diluted 1:10 before injection into the LC-MS system.

LC Conditions

LC system:	ACQUITY UPLC System
Column:	ACQUITY UPLC BEH C_{18} Column, 1.7 µm, 2.1 mm x 100 mm
Mobile phase A:	Water + 0.1% formic acid
Mobile phase B:	Acetonitrile + 0.1% formic acid

Gradient

Time (min)	Flow (mL/min)	%A	%B	Curve
Initial	0.5	100	0	1

Time (min)	Flow (mL/min)	%A	%B	Curve
2.5	0.5	20	80	6
3.0	0.5	20	80	6
3.1	0.5	100	0	6

MS Conditions

MS system:	Q-Tof Premier Mass Spectrometer
Ionization mode:	Electrospray, positive ion
Capillary voltage:	3 kV
Cone voltage:	45 V
Source temp.:	120 °C
Desolvation temp.:	320 °C
Acquisition range:	70–900 amu
Lock mass:	Leucine enkephalin, *m/z*=556.2771

Results and Discussion

Enhanced Duty Cycle

Figure 2 shows extracted ion chromatograms of *m/z*=276 from the analysis of Propanolol in vitro, with EDC turned on and off. The three peaks with retention times at 1.31, 1.39, and 1.47 minutes represent hydroxylated metabolites of Propanolol. There is a ten-fold gain in signal when the EDC is switched on, as compared to when

EDC is off.

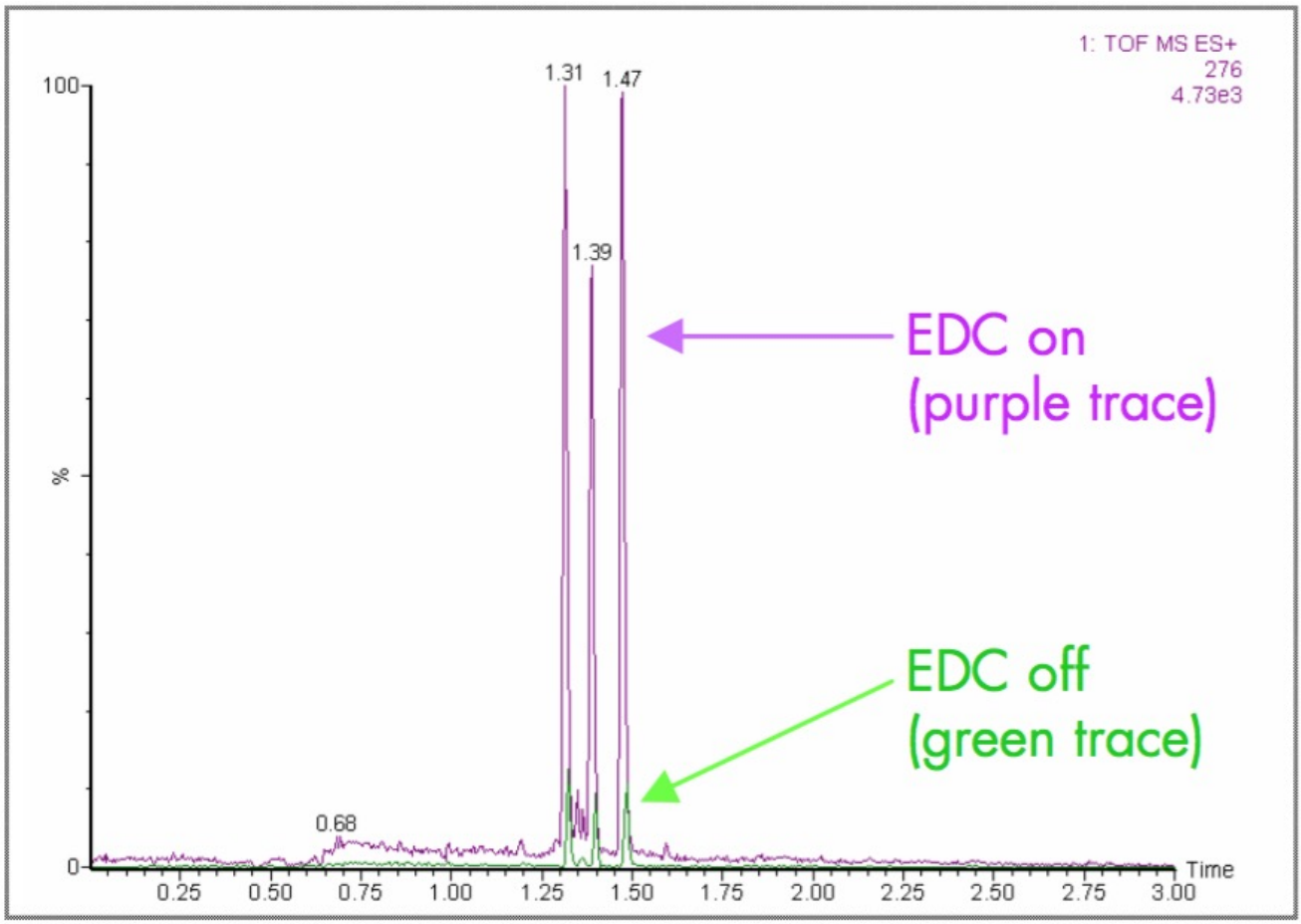

Figure 2. Comparing EDC on and off for a microsomal incubation for Propanolol hydroxylated metabolites.

Figure 3 contains extracted ion chromatograms of *m/z*=630 from the analysis of Propanolol in vitrowith EDC turned on and off to show detection of hydroxylated metabolites of Indinavir. Here, the gain in signal intensity varied between a factor of 2 and 4 with EDC on.

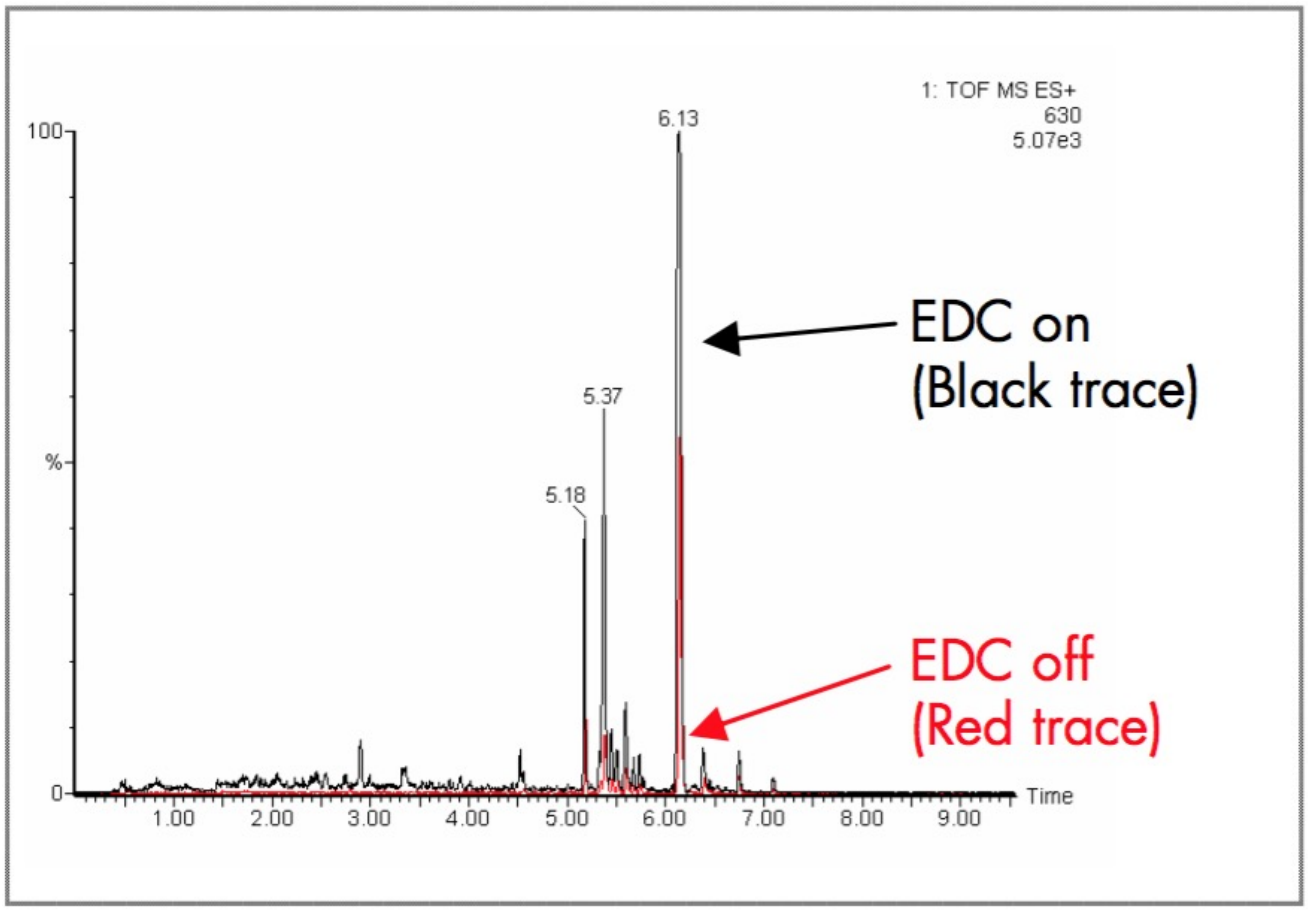

Figure 3. Comparing EDC on and off for rat urine sample for Indinavir hydroxylated metabolites.
The benefits of EDC can be seen in how the low level peaks between retention times 5.37 and 6.13 minutes are magnified. This allows the user to easily detect and verify these metabolites.

MS/MS is also a critical step for metabolite identification. This is where EDC plays a valuable role, when analyzing very low level metabolites to determine the site of biotranformation. Figure 4 illustrates the benefits of EDC for MS/MS applications. Here, it can be observed that the analysis is focused on one of the diagnostic ions for the hydroxylated metabolites of Indinavir. There are several hydroxylated metabolites which will give rise to a product ion at *m/z*=188, most of which are at very low levels making detection and identification difficult. MS/MS is necessary to determine the putative structure of a metabolite. Therefore, the better the sensitivity and the higher the data quality, the easier it will be to determine the correct structure. When EDC is switched on, the signal increases by a factor of 10. The performance advantages of EDC allow the analyst to obtain a much more detailed and information-rich picture of the bioanalytical problem.

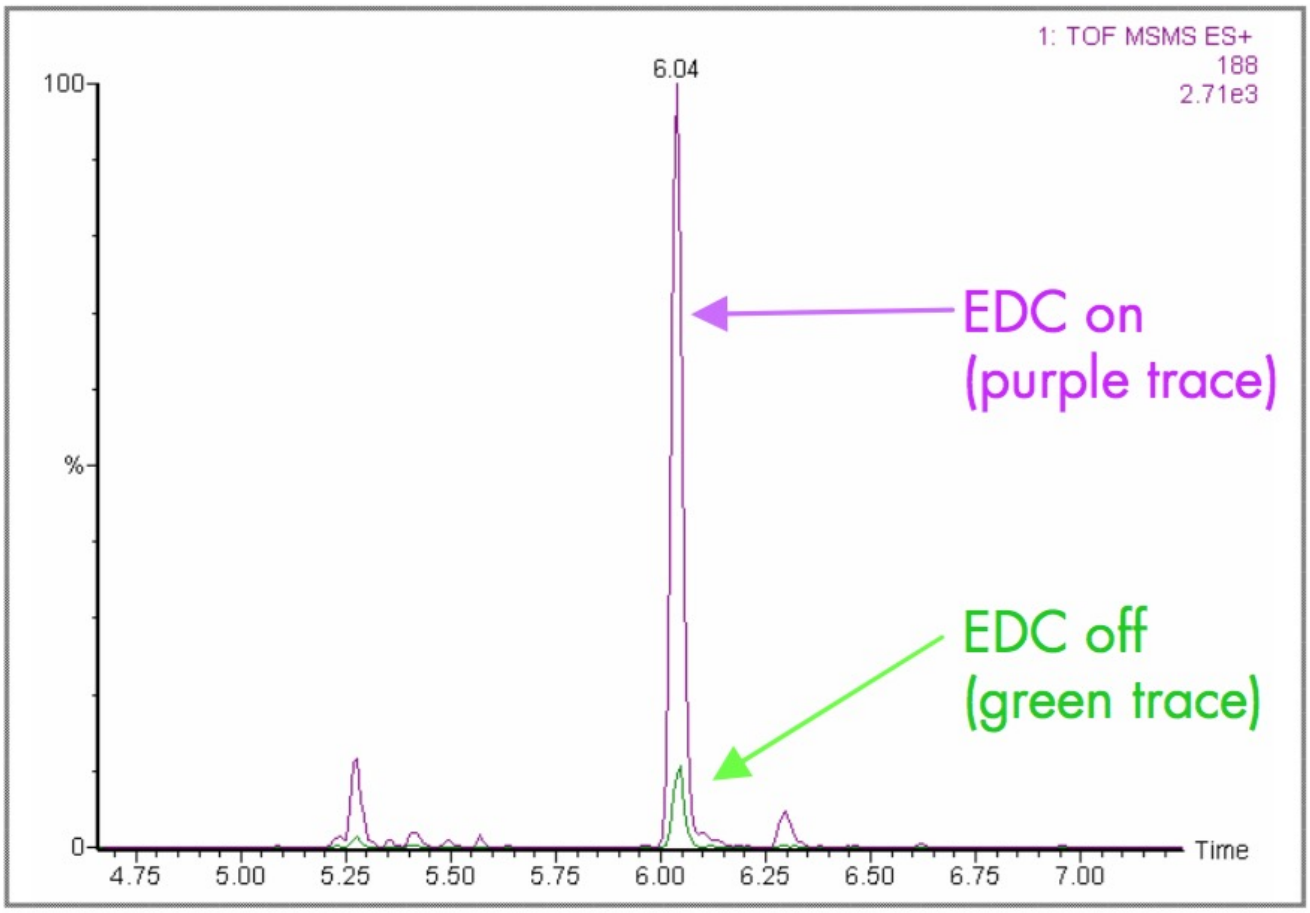

Figure 4. Shows an EDC on and off comparison of m/z=188, a diagnostic ion for the hydroxylated metabolites for Indinavir in rat urine.

Another strategy which may be adopted is to carry out MS/MS acquisitions for targeted diagnostic ions. This will allow the analyst to cross-correlate the results obtained in full scan MS with the targeted MS/MS diagnostics ions, to eliminate the possibility of not detecting important low level metabolites. This is demonstrated with the peak at retention time 6.30 minutes (Figure 4). With EDC switched off it is undetectable, but when EDC is enabled, it is clear that we are able to detect metabolites that we would not have seen otherwise.

Conclusion

This work clearly demonstrates the benefits of using the Q-Tof Premier's EDC functionality as a key analytical strategy to detect very low level metabolites in complex biological matrices. This is very important when the analyst has low volumes of sample available and is therefore limited to the number of LC-MS experiments required to identify the metabolites. EDC may also be used in the highly selective MS/MS mode to focus on a

diagnostic fragment ion when searching for extremely low level metabolites. Overall, EDC provides a higher duty cycle and consequently lower levels of detection for metabolites at low concentrations or those that are poorly ionized.

References

1. The traveling wave device described here is similar to that described by Kirchner in US Patent 5,206,506 (1993).

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