Determination of Butyrate-$^{13}C_4$ in Rat Plasma Using Derivatization and LC-MS/MS Techniques

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INTRODUCTION

Butyric acid is a short-chain fatty acid, esterified in both animal fats and plant oils. Physiological concentrations induce multiple and reversible biological effects. Butyrate is currently under investigation in the treatment of colorectal cancer and hemoglobinopathies. Due to high concentrations of endogenous butyrate, $^{13}C_4$-butyrate analogue was used instead of butyrate for absorption/distribution studies.

The quantitation of butyric acid in plasma by LC/MS method has been challenging because of very weak retention in chromatography and high chemical noise in detection. Here, we explore a derivatization method combined with LC/MS techniques for quantitation of butyrate in rat plasma to enhance chromatographic retention, sensitivity, and selectivity. The method was verified for the robustness and has been used for supporting non-GLP research studies.

METHOD

Following a liquid-liquid extraction procedure, the extract was subjected to a derivatization reaction using *N*-(3-dimethyl aminopropyl)-*N'*-ethylcarbodiimide (EDC) and 2,2,2-trifluoroethyl amine (TFEA) in phosphate buffer (Figure 1). The resulting solution was diluted and chromatographed using a valve switching system consisting of two HPLC columns (Figure 2). Upon initial separation of derivatized butyrate from phosphate salts on an Aquasil C18 column, the fraction was further eluted with a second Aquasil C18 column for enhancing the sensitivity. Butyrate-$^{13}C_4$ and its internal standard (butyrate-d_7) were detected by API 4000 LC/MS system under positive MRM mode.

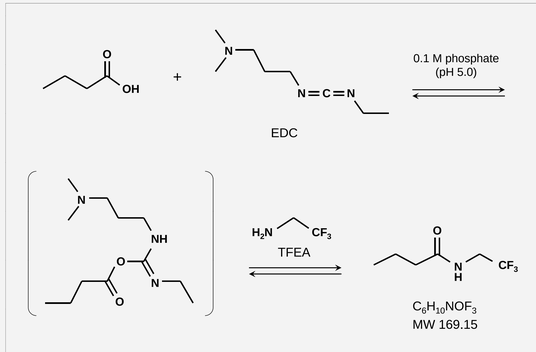

Figure 1. Derivatization Reaction of Butyrate (Reference for derivatization of carboxylic acid: Q. L. Ford et al. *J. Chromatogr. A* 1145 (2007) 241-245)

Liquid Chromatography

HPLC : Shimadzu 10ADvp
Autosampler : Perkin Elmer 200
Column 1: Aquasil C18 (20 x 2.1 mm)
Column 2: Aquasil C18 (30 x 2.1 mm)
MP A : 0.1% Formic acid in DI H_2O
MP B : 0.1% Formic acid in MeOH

Mass Spectrometry

AB Sciex API 4000
Polarity : Positive
Scan type : Multiple Reaction Monitoring
Butyrate-$^{13}C_4$: m/z 174 → 154
Butyrate-d_7: m/z 177 → 157 (I.S.)

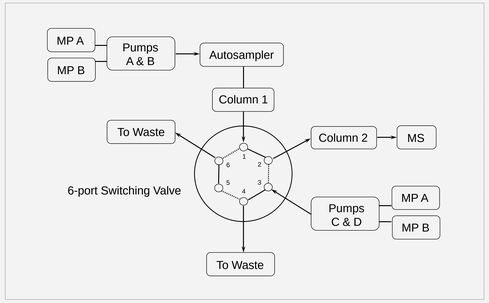

Figure 2. Diagram of LC/MS Instruments.

RESULTS

The method showed a linear range of **4 to 1000 ng/mL** with weighted linear regression (1/x). The correlation coefficients for two verification batches were 0.998 or better (Figure 3).

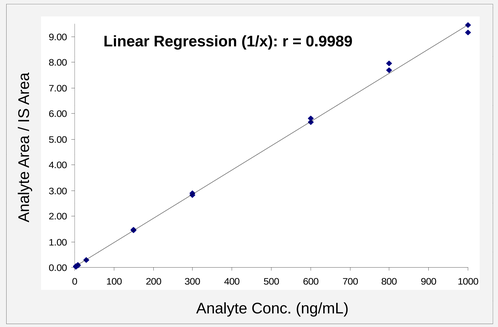

Figure 3. A Typical Calibration Curve for Butyrate-$^{13}C_4$

Figures 4 through 6 show typical chromatograms for selected samples. The S/N ratio for Butyrate-$^{13}C_4$ peak at LLOQ level was greater than 70.

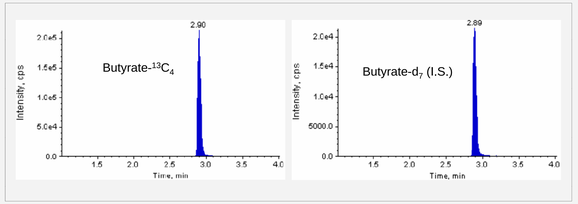

Figure 4. Chromatogram of an Extracted ULOQ Sample (1000 ng/mL).

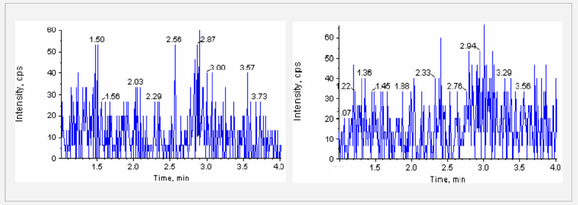

Figure 5. Chromatogram of a Recon Blank (No I.S.) Injected after ULOQ

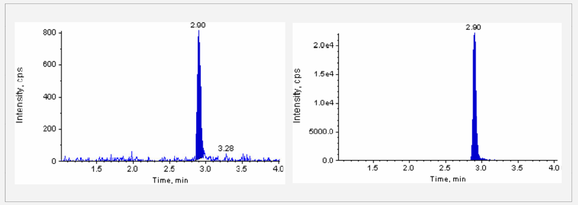

Figure 6. Chromatogram of an Extracted LLOQ sample (4 ng/mL).

Figure 7 shows consistent I.S. responses in a batch indicating the robustness of the derivatization and the method.

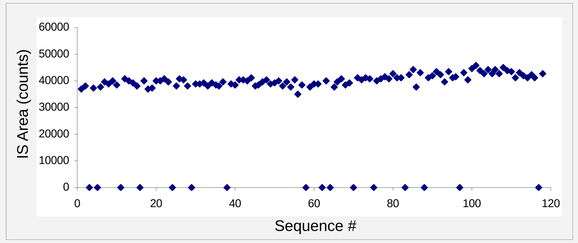

Figure 7. Internal Standard Area Counts in a Batch. (Mean = 40,346 counts, CV% = 5.21%)

The inter- and intra-batch results for QC samples are shown in **Table 1**.

Batch		4.0 ng/mL (LLOQ)	12 ng/mL (Low)	480 ng/mL (Mid)	750 ng/mL (High)
1	**Mean**	4.07	12.5	481	761
	% CV	9.8	4.4	1.5	3.0
	% Bias	1.7	4.5	0.3	1.4
2	**Mean**	4.11	12.5	467	755
	% CV	8.3	7.0	3.1	3.6
	% Bias	2.6	4.5	-2.8	0.7
Overall	**Mean**	4.09	12.5	474	758
	% CV	8.7	5.6	2.8	3.2
	% Bias	2.2	4.5	-1.3	1.1

Table 1. Precision and Accuracy Results for Butyrate-$^{13}C_4$ Quality Control Samples (n = 6 replicates per batch).

	Avg drug area	Avg I.S. area	Avg area ratio
ME 1	4901	39509	0.1241
ME 2	4813	38180	0.1261
ME 3	5078	40518	0.1253
ME 4	4840	39595	0.1222
ME 5	4914	39716	0.1237
ME 6	4898	39259	0.1247
mean	**4907**	**39463**	**0.1244**
n	**6**	**6**	**6**
SD	**92**	**760**	**0.0013**
%CV	**1.9**	**1.9**	**1.1**

Table 2. Matrix Effect for Butyrate-$^{13}C_4$ in Rat Plasma.

CONCLUSION

A reliable method has been established for the measurement of $^{13}C_4$-butyrate in rat K_2EDTA plasma samples, using LC-MS/MS techniques with butyrate-d_7 as the internal standard. The combination of a simple sample cleanup procedure with a selective derivatization procedure was effective in eliminating high chemical noise. Further, the resulting trifluoroethylamide derivatives improved both chromatographic performance and linear dynamic response. This coupled with column valve switching techniques for the removal of derivatization salts contributed to the robustness of the assay. This method has shown acceptable accuracy, selectivity, and reproducibility. It was successfully applied to the analysis of pre-clinical samples.

ACKNOWLEDGEMENTS

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