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ScienceDirect
Journal of Chromatography B, 854 (2007) 57–62
JOURNAL OF CHROMATOGRAPHY B
www.elsevier.com/locate/chromb

Application of liquid chromatography–mass spectrometry to measure the concentrations and study the synthesis of short chain fatty acids following stable isotope infusions

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Received 5 January 2007; accepted 29 March 2007
Available online 8 April 2007

Abstract

A new method involving zinc sulphate deproteinization was developed to study short chain fatty acids (SCFA) production in the colon and subsequent occurrence of SCFA in blood. SCFA were baseline separated in a 30 min cycle using ion-exclusion chromatography and detected by mass spectrometry. Concentrations could be measured down to 10 μM and isotopomeric distributions could be assessed, enabling the conduction of tracer studies to study changes in SCFA synthesis. The applicability of the method was tested in an extensively characterized pig model yielding portal SCFA concentrations ranging from 70 μM (butyric acid) to 150 μM (propionic acid) to 440 μM (acetic acid) prior to butyrate tracer infusion, reaching butyric acid isotopic steady state within 2 h.

Keywords: Short chain fatty acids; LC–MS; SCFA; Stable isotope

1. Introduction

The volatile carboxylic acids, acetic acid, propionic acid and butyric acid (usually referred to as "short chain fatty acids; SCFA) are produced along with other substrates, such as lactate, methane, carbon dioxide and ethanol during microbial fermentation of non-digestible carbohydrates as well as endogenous substrates (e.g. mucus), in the gastrointestinal tract [1]. It is known that SCFA are of high importance for colonic health maintenance [2,3], however the kinetics of their production and metabolism in the colon in vivo, the major production site in humans, are still unclear. In general, SCFA concentrations appear to depend mostly on the size and diversity of the colonic bacteria population, as well as the time and extent to which digests are retained in the colon, in addition to the type of carbon source [4,5].

A better understanding of the mechanisms underlying gut microbial SCFA production may make it possible to manipulate SCFA concentrations in the colon by modifying for instance nutrition, use of pre- and probiotics and medication. To investigate this, we needed to be able to measure SCFA concentrations and determine synthesis rates in vivo. To study the regulatory mechanisms of metabolic pathways in vivo, stable isotope tracer infusion studies are frequently used [6,7]. For the present study we focussed on the kinetics of butyric acid applying a sodium-[1-^{13}C]-butyric acid tracer in a well-characterized pig model [8]. Butyric acid is an important energy source for colonocytes and acts as a health-mediating compound and as such (changes in)

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doi:10.1016/j.jchromb.2007.03.044

colonic concentrations or more preferably the in vivo bacterial synthesis rate are of a high importance. Pigs were chosen for these studies because the digestive physiology of this animal is in many ways comparable with the human digestive physiology [8], implicating that they also produce relatively high SCFA concentrations in the large intestine [9]. The pig model used, allowed the simultaneous sampling of portal venous, hepatic venous and arterial blood in conscious pigs [8], and was adapted with a stoma for the direct infusion of sodium-[1-^{13}C]-butyrate tracer into the caecum. As SCFA are volatile components, most of the published methods use gas chromatography (GC) either with or without mass spectrometry (MS). The derivatization procedures involved in these GC based methods, require the (laborious) extraction into an organic solvent and this process requires the addition of acid to the plasma matrix [7,10]. However, lowering the plasma pH also promotes the evaporation of SCFA, thus compromising their recovery. Sample preparation is thus a critical issue to obtain reliable results.

Considering the above, we developed a new method, minimizing sample preparation and utilizing liquid chromatography–mass spectrometry (LC–MS) to measure SCFA concentrations and their isotopomeric distribution to enable the setup of stable isotope based tracer infusion studies.

2. Materials and methods

2.1. Chemicals

All chemicals used were of analytical grade unless specified otherwise, purchased mostly from Sigma–Aldrich (Zwijndrecht, Netherlands). Stable isotope tracers, e.g. sodium-[1-^{13}C]-acetate (99%), sodium-[1-^{13}C]-propionate (99%) and sodium-[1-^{13}C]- butyrate (99%), were all purchased from ARC Technologies (Apeldoorn, the Netherlands). Ultra pure water was generated through a Super-Q water purification system (Millipore, Amsterdam, the Netherlands).

The infusate consisted of an isotonic (300 mOsm) solution of 6 mM sodium [1-^{13}C]-butyrate in saline. Before infusion, stable isotope infusion solutions were sterilized by filtration through a 0.22-μm sterile filter.

2.2. Liquid chromatography–mass spectrometry

The chromatographic system consisted out of a Model PU-980 HPLC pump (Jasco Benelux, Maarsen, the Netherlands) and a Model 717 WISP sample processor (Waters, Etten-Leur, the Netherlands) equipped with a cooled sample storage compartment (10 °C). The analytical column was an IOA-1000 ion-exchange column (300 mm × 7.8 mm I.D.), Grace-Alltech (Breda, the Netherlands) mounted in a column oven, set to 90 °C. Separation of SCFA was achieved by isocratic elution using an aqueous solution of benzoic acid (0.15 mM) at 0.6 mL/min. The mass spectrometer was a Model LCQ Classic (Thermo Electron, Breda, the Netherlands), equipped with an atmospheric pressure chemical ionization (APCI) probe. The mass spectrometer was operated in negative mode, using the selected ion monitoring scan function (SIM), set to a range of *m/z* 50–99. Maximal sensitivity of the LC–MS system was obtained at the following settings: 250 ms ion time; 5 micro scans; vaporizer temperature: 230 °C; heated capillary temperature: 90 °C; corona discharge current: 10 μA. To avoid rapid contamination of the heated capillary, the column effluent was not introduced in the mass spectrometer during the first 13 min after injection. In between injections, the mass spectrometer probe was flushed with an aqueous solution of benzoic acid (0.15 mM) supplied by a second Model PU980 pump at 0.6 mL/min. After elution, the column effluent was mixed with 0.1 mL/min of aqueous ammonia (0.13 M) by a third Model PU-980 HPLC pump, using a peek-mixing tee, raising the column effluent pH to about 9–10, thus supporting SCFA ionization efficiency and subsequently enhanced sensitivity.

2.3. Animal experiments

An extensively characterized pig model [8] was adapted with a Bishop-Koop stoma placed at the caecum to allow direct infusion of tracer solutions into the colon and sampling of the colonic metabolites. All details on animal procedures are described in [8].

Before the start of sodium-[1-^{13}C]-butyrate tracer infusion, background blood samples were collected and thereafter a caecal infusion of the sodium-[1-^{13}C]-butyrate solution at a constant rate (12 μmol/kg/h) via the stoma was applied for 240 min after which a bolus injection of 37.8 μmol/kg was applied directly into the caecum (i.e. via the stoma). Hereafter, the tracer infusion was stopped. Portal venous, hepatic venous and arterial blood samples were taken every 30 min from the start of the tracer infusion until 60 min after the bolus injection.

Portal venous, hepatic venous and arterial blood samples collected by the pre-implanted catheters [8] were transferred into 1.3 mL micro Li-heparinized blood sample tubes (Sarstedt, Etten-Leur, The Netherlands) cooled on ice. Blood samples were immediately centrifuged at 8500 × *g* in a Model Hereaus Biofuge Stratos (Dijkstra Vereenigde, Lelystad, The Netherlands) for 10 min at 4 °C. Plasma was separated and then transferred into 2 mL safe lock micro tubes (VWR, Amsterdam, The Netherlands) and stored at −80 °C until analysis. Before analysis, 150 μL plasma aliquots transferred into 1.5 mL safe-lock micro tubes were deproteinized either by addition of 15 μL zinc sulphate solution (4.6 g/10mL water), 15 μL of trichloroacetic acid (TCA) solution (5 g/10 mL water) or 9 mg of solid 5-sulphosalicylic acid (SSA), vortex-mixed vigorously and centrifuged at 50.000 × *g* for 10 min at 4 °C. A 125 μL aliquot of the clear plasma supernatant was transferred into a 300 μL glass micro-insert (40 mm × 6 mm), spring adjusted into a 4 mL WISP-style vial (Waters, Etten-Leur, Netherlands) and sealed with a PTFE seal. A 100 μL aliquot was injected into the LC–MS system.

2.4. Calculation of tracer–tracee ratio (TTR)

Many elements have one or more stable isotopes, which have the same chemical properties but are distinguished from

each other by a difference in weight, a property which can be measured using a mass spectrometer. These isotopes can be used to "tag" a molecule by replacing an atom by its heavy isotope. The tracer–tracee ratio (TTR) is one of the most commonly used expressions to describe the incorporated amount of stable isotope tracer resulting e.g. from the supply of tracer through for instance infusion [11]. Here, we will refer to the isotopomeric peaks of a given molecule (with mass M;) as $M+1$ (containing one heavy isotope), $M+2$ (containing 2 heavy isotopes) and so on. By natural occurrence, molecules already contain nature-defined amounts of "tracer" atoms (e.g. ^{13}C) and thus a TTR is calculated by dividing the concentration of the tracer (at $M+1$, $M+2$, etc.) by the concentration of the tracee:

$$\mathrm{TTR}_{\mathrm{natural}}\,(\%) = \left(\frac{[\mathrm{tracer}]_{\mathrm{natural}}}{[\mathrm{tracee}]_{\mathrm{natural}}}\right) \times 100\%$$

When a tracer is supplied, its concentration will increase relative to the concentration of the tracee and thus the TTR will increase according to:

$$\Delta\mathrm{TTR}\,(\%) = \mathrm{TTR}_{(\mathrm{post\ infusion})}\,(\%) - \mathrm{TTR}_{(\mathrm{pre\ infusion})}\,(\%)$$

This ΔTTR can be used to calculate changes in ongoing physiological processes.

3. Results

3.1. Influence of deproteinization agent

To investigate the effects of the deproteinization agent on SCFA recovery, 200 μL pig plasma was spiked with 10 μL of an 1 mM SCFA solution (10 nmol abolute of each SCFA) and spiked and non-spiked aliquots were treated with SSA, TCA and zinc sulphate. On average ($n=5$), the use of TCA resulted in about 30% recovery; SSA about 60% recovery while zinc sulphate gave about 100% recovery (Fig. 1).

3.2. System optimization

To minimize mass spectrometer (MS) noise to a level as low as possible we first used pure water to elute SCFA from the IOA-1000 ion-exchange column. Using this approach we were able to separate aqueous SCFA standards and obtained low MS-noise levels. However, it was not possible to obtain a reproducible separation for (deproteinized) plasma samples, probably due to presence of salts in the sample. To stabilize this separation we tried various solvent concentrations of pivalic acid (pK_a 5.03) and benzoic acid (pK_a 4.19). Benzoic acid provided a stable separation at a concentration of only 0.15 mM, whereas for pivalic acid a 5–10 times higher concentration was required. But pivalic acid application at these high concentrations gave a higher MS noise level and therefore SCFA analysis was done using benzoic acid.

By setting the column temperature to a maximal 90 °C, baseline separations were obtained for all SCFA of interest at retention times of 16.0 min (acetic acid), 18.1 min (propionic acid) and 20.5 min (butyric acid) (Fig. 2). Under these conditions, retention efficiency guarantees a good separation between coextracted salts and analytes of interest. In addition, to support SCFA ionization in negative mode, the column effluent was mixed with 0.13 M ammonia at a flow of 0.1 mL/min to raise the pH to ∼9.5 before entering the MS. This addition resulted in a 2–3 times increase in signal strength solution.

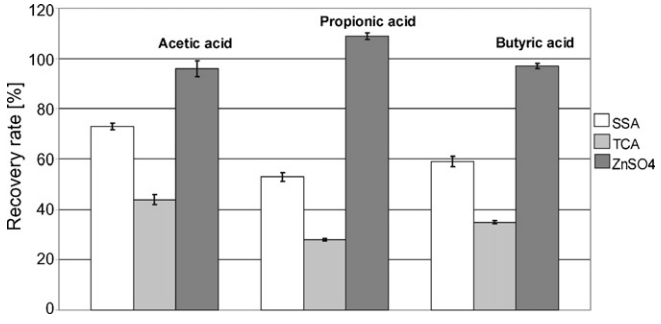

Fig. 1. Recovery rates of SCFA from spiked (50 μM) portal pig plasma with different deproteinization agents. Data are presented as mean ± relative standard deviations (RSD).

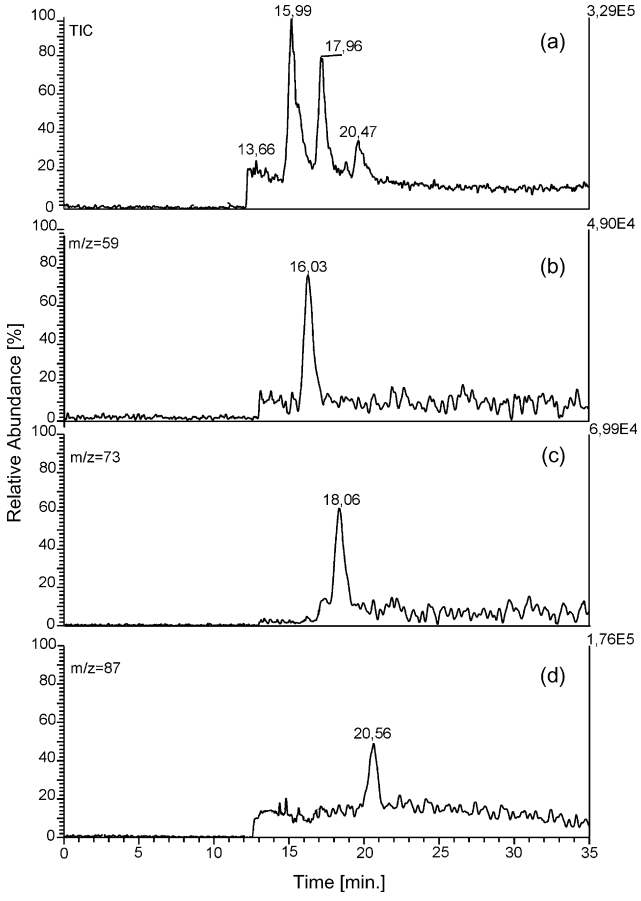

Fig. 2. LC–MS chromatogram of selected ions ($m/z=59$, 73 and 87) from (a) portal plasma sample at $t=240$ min; (b–d) selected ion trace of a SCFA standard solution (12.5 μM), acetic acid ($m/z=59$) (b); propionic acid ($m/z=73$) (c); and butyric acid ($m/z=87$) (d). Measured SCFA concentrations were 244, 75 and 97 μM for acetic-, propionic- and butyric acid, respectively. For LC–MS conditions see Section 2.

Table 1
Validation parameters of the method

Validation parameter	Analyte		
	Acetic acid	Propionic acid	Butyric acid
Target analyte ions $(M-H)^-$	59, 60	73, 74	87, 88
Concentration range (μM)	10–250	10–250	10–250
Regression coefficient (r^2)	0.9991	0.9995	0.9991
Regression line equation	$y = 76473x + 56143$	$y = 106742x + 142612$	$y = 171493x + 178165$
Limit of detection (μM)	7.5	7.5	7.5
Relative standard deviation (RSD, $n = 3$, %)	2.8	3.7	2.4

3.3. Calibration standards

SCFA concentrations were determined through the external standard method. Signal linearity of the LC–MS response was studied in triplicate using aqueous calibrators of 10, 25, 50, 100 and 250 μM, respectively, prepared from pure chemicals and plotting the concentrations against the summarized peak areas of the $[M-H]^-$and $[M+1]^-$ isotopes of each SCFA. Calibration curves were linear in the studied concentration range (r^2 of all SCFA > 0.99; Table 1). The limit of detection (LOD) for acetic acid and propionic acid was 10 μM while the LOD for butyric acid was 10 μM, calculated at a minimum signal-to-noise ratio (S/N) of 3:1.

The accuracy of the TTR measurement was studied and validated by aqueous calibrator solutions prepared from pure sodium-[1-^{13}C]-tracers. Solutions with TTR ratios of 0, 5, 10, 25, 50, 75 and 100%, above the natural TTR of each SCFA at two different concentration levels, 50 and 100 μM, respectively were used to study the accuracy of the TTR measurement.

For the 50 and 100 μM level in all cases correlation coefficients $r^2 > 0.99$ were obtained (Table 2). Measured TTRs were plotted against theoretical TTRs. Calibration lines of the TTR determination were calculated at both concentrations by linear regression analysis. Equations of the TTR calibration curves at the 50 μM concentration level were $y = 1.015x - 0.402$, $y = 1.011x - 0.225$ and $y = 0.979x + 0.363$ for sodium-[1-^{13}C]-acetate, sodium-[1-^{13}C]-propionate and sodium-[1-^{13}C]-butyrate, respectively. At the concentration level of 100 μM equations of the TTR calibration curves were $y = 0.942x + 3.441$, $y = 1.02x - 0.97$ and $y = 0.994x - 0.617$ for sodium-[1-^{13}C]-acetate, sodium-[1-^{13}C]-propionate and sodium-[1-^{13}C]-butyrate, respectively.

The mean levels and variation of the TTRs (natural) were determined at different SCFA concentrations 25, 50, 100 and 250 μM. Relative standard deviations (RSD) increased with decreasing concentrations (0.3, 0.2, 0.1 and 0.03% for SCFA concentrations of 25, 50, 100 and 250 μM, respectively).

3.4. Plasma samples

The target for this and future physiological experiments was to see if we could determine changes in concentrations of SCFA and/or TTR in time. For this goal SCFA concentrations were determined in portal pig blood samples taken at different time points. The results are shown in Fig. 3. Clearly, the present method provides more than sufficient sensitivity, as the lowest concentrations measured are still a factor of 10 above the LOD (Table 1).

In this experiment, [1-^{13}C]-butyric acid was infused in the caecum. The time profiles of acetic acid and propionic acid concentrations showed a fluctuating behavior with a gradual decrease in time. In contrast, butyrate initially remained at a more or less stable level, then increased from $t = 45$ to

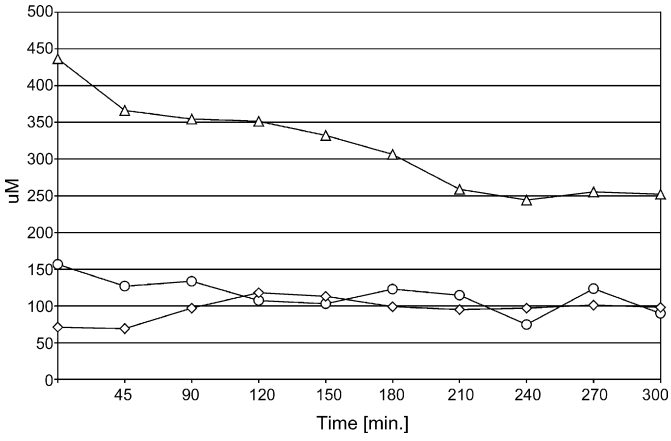

Fig. 3. Concentrations of acetic acid (△), propionic acid (◯) and butyric acid (◇) in pig portal plasma samples.

Table 2
Regression coefficients of tracer–tracee ratio calibration curves

Analyte	Concentration 50 μM		Concentration 100 μM	
	Regression coefficient (r^2)[a]	Regression line equation	Regression coefficient (r^2)[a]	Regression line equation
[1-^{13}C]-acetic acid	0.9996	$y = 1.02x$	0.9987	$y = 0.94x$
[1-^{13}C]-propionic acid	0.9999	$y = 1.01x$	0.9985	$y = 1.02x$
[1-^{13}C]-butyric acid	0.9996	$y = 0.98x$	0.9995	$y = 0.99x$

[a] Triplicate determination.

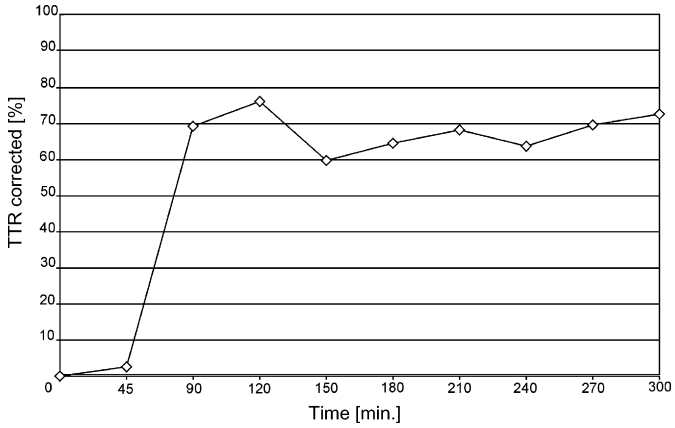

Fig. 4. Measured ΔTTR (%) for [1-^{13}C]-butyric acid in pig portal plasma samples in time.

210 min, decreasing thereafter to a steady state value of around 100 μM.

Results from the infusion experiment of the butyrate tracer, showed that the plasma 1-[^{13}C]-butyric acid concentration increased rapidly to a plateau at approx. 70 μM while the non-enriched tracee remained stable at approx. 100 μM resulting in a corrected TTR (plateau) for its natural TTR of around 65% in an apparent steady state of the butyric acid pool (Fig. 4).

4. Discussion

Most presently known methods for the determination of SCFA concentrations and/or TTR measurements in plasma are based on GC–MS or GC–C–IRMS techniques [7,12,13]. In general, the reported methods are laborious due to a complex sample preparation (e.g. extraction, derivatization, etc.). Sample preparation, extraction and derivatization are however critical steps as they may easily influence analyte recoveries. To exclude some of these variables and guarantee reliable results of SCFA production and concentration in in vivo studies with pigs, we developed an alternative approach to the already available gas chromatography based methods. One of the most important aspects of sample preparation of plasma samples is the removal of proteins. Acids commonly used for this purpose [14] lower the pH below the pK_a of SCFA, resulting in their protonation, thus promoting evaporation from the sample matrix, which may result in variable recoveries. The expectation was that the lower the pH (due to addition of a stronger acid), the more pronounced the effect would be. Indeed this could be confirmed by applying two different acids TCA and SSA to plasma samples spiked with known amounts of SCFA (Fig. 1). Organic solvents could normally provide an alternative for deproteinization, but may influence the retention behavior when using reversed phase chromatography. Unfortunately in the present application it cannot be applied at all as the ion-exchange column used in this application does not tolerate any organic solvents. Surprisingly, zinc sulphate is hardly ever considered a good deproteinization alternative, although it has been successfully employed for the determination of a range of substances in plasma [15]. In this application, it also provided an easy to use alternative with a nearly 100% recovery for all SCFA (Fig. 1).

To set up an LC–MS measurement of SCFA in blood, various liquid chromatography methods were available, including reversed phase chromatography [16,17] and ion-exclusion chromatography [18]. However, a pre-requisite for an LC–MS method is that the solvents applied are volatile. However, most reversed phase methods employ inorganic buffers (e.g. phosphate buffers), and as the targeted SCFA in this application are acids that usually are used to replace phosphate buffers in LC–MS, it is not possible to use these acids. Theoretically, ion exchange chromatography seemed to provide an alternative because even pure water could be applied as solvent. And for aqueous standards this indeed seemed to work (data not shown). However, probably due to high salt loads, for deproteinized plasma samples, the addition of acid to elution solvents was required to stabilize retention times and improve peak shape. This raised the question what acid should be used. The ideal candidate should be volatile, not containing target analytes contamination and should preferably have a pK_a value not too much below the pK_a of the SCFA (about 4.8) as this would promote protonation, while SCFA can only be measured in negative mode. Considering these requirements we tried benzoic acid (pK_a 4.19) and pivalic acid (pK_a 5.03). From these, benzoic acid provided the best results, both with respect to retention behavior and peak shape, as well as the lowest noise level in the MS measurement. A concentration of 0.15 mM was found to be optimal, as a higher concentration would provide an even better peak shape and shorter retention times, but increased MS noise in a disproportional way (not shown).

Considering the analytes elute in a buffer, which is below their pK_a value, they are predominantly in the protonated state, thus resisting negative ionization as required for their entrance in the MS system. Therefore, prior to introduction into the MS system, the column effluent was mixed with an ammonia solution (0.13 M) by a peak mixing tee using a second HPLC pump. In this way, the column effluent pH was increased to about 9–10. As a consequence, the MS response of the analytes was raised by a factor of approximately 2–3.

The present method allowed the baseline separation of all three SCFA (Fig. 2), at retention times of 16.0 min for acetic acid, 18.1 min for propionic acid and 20.5 min for butyric acid, respectively. Before 13 min, the column elution was directed to the waste, avoiding the introduction of some coextracted salts in the source.

Using the above described method, plasma samples were analyzed that were collected in a pilot tracer infusion study performed in a pig. Measured SCFA concentrations in portal pig plasma ranged between 80 and 430 μM (Fig. 3). However, following the portal concentrations of SCFA in time, we observed a gradual decrease (Fig. 3). While the continuous decrease in the portal acetic acid concentration may reflect a steadily decreasing production rate in the caecum due to gradual exhaustion of the carbon sources in the colon, more experiments are needed to confirm this hypothesis. The propionic acid concentration profile shows an identical trend (Fig. 3). However after 120 min, the portal propionic acid concentration no longer decreased but

started a fluctuating behavior. Also in this case more experiments are needed to assess the reproducibility of this observation.

In contrast to the acetate and propionate profiles, the butyric acid concentration started to increase after $t = 60$ min (Fig. 3). This rise coincided with a rise in the butyric acid TTR (Fig. 4). The interpretation of this behavior is, first, that there was an apparent delay between the start of infusion and the appearance of butyrate in the portal plasma. This delay probably reflects the time needed by the infused butyrate to mix and/or diffuse throughout the caecal compartment. Secondly, the chosen butyrate infusion rate of 0.2 μmol/kg/min apparently caused a marked increase of the caecal concentration of this SCFA, leading also to increase portal plasma concentrations. This corroborates the fact that the measured TTR was between 60 and 80%, indicating that the infusion rate was of the same order of magnitude as the colonic butyrate synthesis rate, which would mean an approximate doubling of butyrate appearance in the colon hence also in the portal circulation. Surprisingly, the sodium-[1-^{13}C]-butyrate bolus of 37.8 μmol/kg/h at $t = 240$ min did not produce a significant rise in portal blood butyric acid concentration and produced only a minor increase in butyrate TTR. This might be caused by a similar delay for mixing and/or diffusion as observed at the start of the experiment.

The experimental data do provide insight in pig butyrate metabolism. First, from additional concentration measurements in hepatic venous blood, it will be possible to quantitate the contribution of the liver to SCFA metabolism in vivo. Secondly, from the average butyric acid TTR in steady state between $t = 150$ and 240 min of around 65% (Fig. 4), a colonic butyrate production rate of I/TTR = 0.2/0.65 = 0.3 μmol/kg/min is calculated, assuming complete mixing of the infusate with the intraluminal colonic butyrate pool. This compares well with values reported in Kien et al. [19] for pigs, which varied between 0.4 and 2.8 μmol/kg/min, however in a different pig strain and during continuous enteral infusion of lactulose and lactose at a rate of around 19 μmol/kg/min.

It is expected that these and similar observations, enabled by the newly developed analytical procedure, will contribute significantly to our understanding of SCFA metabolism in the interplay between host and microbes in the near future.

5. Conclusion

The newly developed method is suitable for the analysis of SCFA concentrations and TTR in pig plasma. The present paper demonstrates the application of a protocol to study SCFA metabolism in the pig using caecal butyrate infusion and sampling of portal venous, hepatic venous as well as abdominal arterial blood compartments. Further interpretation of the observed data indicates that the new LC–MS protocol should first be applied in a full multi-organ study that includes material balances over the gut, the liver, and the peripheral circulation.

Acknowledgement

The authors like to thank Mr. D.P.L. Suylen for his excellent technical assistance.

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