Available online at www.sciencedirect.com
SCIENCE DIRECT®
Journal of Pharmaceutical and Biomedical Analysis
32 (2003) 513–522
JOURNAL OF PHARMACEUTICAL AND BIOMEDICAL ANALYSIS
www.elsevier.com/locate/jpba

Shift in pH of biological fluids during storage and processing: effect on bioanalysis

Aberra Fura *, Timothy W. Harper, Hongjian Zhang, Lawrence Fung, Wen Chyi Shyu

Bristol Myers Squibb, Pharmaceutical Research Institute, P.O. Box 5400, Princeton, NJ 08534, USA

Received 10 September 2002; received in revised form 29 January 2003; accepted 5 February 2003

Abstract

The pH of ex vivo plasma, bile and urine was monitored at different times and temperatures of storage, and following different sample processing methods such as ultrafiltration, centrifugation, precipitation and evaporation. The results showed that the pH of ex vivo plasma, bile and urine increased upon storage, and following sample processing and could lead to significant degradation of pH-labile compounds. Several compounds were used to illustrate the impact of pH shifts on drug stability and interpretation of results obtained from in vivo studies. For example, after 1 h of incubation (37 °C) in rat plasma (pH 8.3), about 60% of **I** was lost. However, in phosphate buffer, losses were about 12% at pH 7.4 and 40% at pH 8.0. Plasma pH also increased during ultrafiltration, centrifugation and extraction. After methanol precipitation of plasma proteins, and evaporation of the supernatant and redissolution of the residue, the resulting solution had a pH of 9.5. Under these conditions, **II** was degraded by 60% but was stable when phosphate buffer was used to maintain the pH at 7.4. The shift in plasma pH can yield misleading results from in vivo studies if the pH is not controlled. For example, the major circulating metabolite of **II** was also formed in plasma ex-vivo. This ex vivo degradation was prevented when blood samples were collected into tubes containing 0.1 volume of phosphate buffer (0.3 M, pH 5). The pH of ex vivo plasma can best be stabilized at physiological conditions using 10% CO_2 atmosphere in a CO_2 incubator. Changes in pH of ex vivo urine and bile samples can have similar adverse effect on pH-labile samples. Thus, processing of plasma samples under a 10% CO_2 atmosphere is a method of choice for stability or protein binding studies in plasma, whereas citrate or phosphate buffers are suitable for stabilizing pH in bile and urine and for plasma samples requiring extensive preparations or long term storage.

Keywords: Ex vivo plasma pH; Ex vivo bile pH; Ex vivo urine pH; pH stabilization; pH dependent degradation

1. Introduction

During pharmacokinetic and/or metabolism studies, drug and drug metabolite levels are commonly monitored in plasma, urine or bile samples. In these studies, samples are generally

* Corresponding author. Tel.: +1-609-818-4274; fax: +1-609-818-3675.
E-mail address: aberra.fura@bms.com (A. Fura).

0731-7085/03/$ - see front matter © 2003 Elsevier Science B.V. All rights reserved.
doi:10.1016/S0731-7085(03)00159-6

collected, stored for a period of time, processed in some manner, and then analyzed. Sample processing generally may involve ultrafiltration, centrifugation and/or concentration through evaporation to dryness and redissolution in an appropriate solvent. These steps may lead to changes in the pH of the ex vivo samples from the initial physiological pH.

The physiological pH of plasma in the body is kept essentially constant between 7.35 and 7.45, despite the daily generation of over 12 moles of CO_2 and other acids produced by the metabolism of food. Metabolizable acids and bases from the diet, acidic or basic groups on larger molecules such as proteins, and non-metabolizable acids and bases such as NaOH and H_3PO_4 from the diet or as byproducts of metabolism also affect the pH of plasma. These effects are regulated by the rate of metabolism and by renal mechanisms. However, the most important buffer system for maintaining the physiological pH is the carbonic acid–bicarbonate system whose equilibrium is controlled by the rate of pulmonary ventilation [1]. Ex vivo blood or plasma does not have the pH regulatory mechanisms described above. In addition, the buffering capacity of the carbonic acid–bicarbonate system in ex vivo blood or plasma is compromised as CO_2 is continuously lost upon standing or storage, and during sample processing. As a result, the pH shifts from the physiological pH, becoming increasingly more alkaline within few hours after collection.

Important phenomena such as binding of drugs to plasma proteins are often pH dependent. As a result, any change in the ex vivo plasma pH can significantly affect protein-binding measurements [2–6]. Chemical stability of compounds can also be adversely affected by a change in pH [7]. For example, compounds containing simple esters and amides, carbamates, urea, β-lactam, lactones, hydantoines, acyl glucuronides etc. are known to be prone to pH dependent degradation. A change in ex vivo plasma pH can, therefore, have a significant effect on measured drug concentrations of pH-labile compounds. This paper reports systematic studies of the effect of pH shifts in ex vivo plasma on drug stability and drug disposition studies and describes methods that can be used to stabilize the ex vivo pH of plasma.

This paper also discusses the changes in pH of bile and urine samples during sample storage and processing and the importance of controlling this pH change when measuring drug levels. As mentioned above, analyses of bile and urine samples are important components of the drug disposition studies, since biliary or urinary excretion routes are often major elimination pathways of drugs and drug metabolites.

Bile is a complex mixture containing bile salts, fats, fatty acids and inorganic salts. It is generally alkaline due to its bicarbonate content and, like plasma, may be expected to exhibit ex vivo pH changes resulting from loss of CO_2. As with plasma, analyses of pH-sensitive compounds may be significantly affected by any ex vivo changes in the pH of bile samples. For example, biliary excretion is a common route of elimination of metabolites such as glucuronides, and it is known that many acylglucuronides undergo degradation or chemical transformation in a pH dependent manner [8].

Urine samples have a wide range of pH (pH $\sim$ 4–8) [9] depending on the diet, medications, and health status of the individuals. Since urine samples contain, among other things, salts such bicarbonates, phosphates and ammonium salts, the pH of urine samples may change during storage or processing, resulting in difficulties with quantitation of pH-labile compounds.

2. Materials and methods

2.1. Solvents and chemicals

Sodium citrate ($Na_3C_6H_5O_7 \cdot 2H_2O$), citric acid monohydrate, sodium phosphate (both monobasic and dibasic), ammonium acetate, acetic acid, phosphoric acid, sodium hydroxide, and rat albumin were obtained from Sigma. HPLC grade acetonitrile and methanol were obtained from EM Science. Compounds **I**, **II**, **III** (Fig. 1) were obtained from Discovery Chemistry, Bristol-Myers Squibb, Pharmaceutical Research Institute.

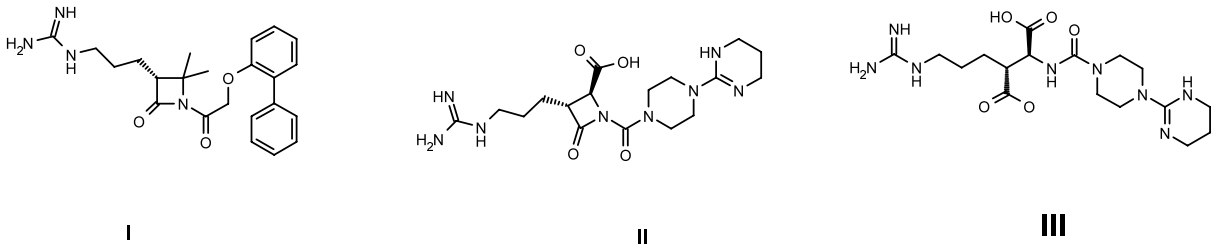

Fig. 1. Chemical structures of compounds studied.

2.2. Plasma, bile and urine, lung and gastrointestinal tissue

Plasma samples were obtained from naive rats after an overnight fast or from rats receiving **II**. For the naïve rats, blood samples were collected either after CO_2 asphyxiation or after treatment with the anesthetic isofluorane. For the three rats receiving a 10 umol/kg dose of **II** intraarterially, blood samples were collected via a jugular vein cannula at scheduled time points. All blood samples were collected into tubes containing a pertinent anticoagulant and the plasma was prepared by centrifugation (2500 rpm for 15 min, 4 °C). Bile samples were collected from bile duct cannulated (BDC) naive rats after an overnight fast. Lung and gastrointestinal tissues were collected from overnight fasted rats, and the homogenates were prepared with water. Animal handling and treatment were carried out in accordance with Bristol-Myers Squibb policy.

2.3. pH measurement

The measurement of pH was carried out using a Beckman pH meter equipped with a Beckman Futura long combination pH electrode. The pH meter was calibrated using commercially available two-point standard buffers before every experiment. The pH of the plasma samples was determined before and after the plasma samples were treated with a small amount of buffer (citrate or phosphate, see below). Under these conditions, the plasma samples were capped tightly and incubated at different time points and temperatures (ambient or 37 °C water bath) and opened only during the pH measurement. In addition, pH values were measured in plasma samples stored under a carbon dioxide atmosphere for various periods of exposure. These plasma samples were stored in loosely capped tubes and subjected to carbon dioxide atmosphere (5 to 10% CO_2) in a CO_2 incubator (NAPCO Water Jacketed CO_2 incubator, Precision Scientific Inc) at 37 °C. The pH of ex vivo plasma samples was also measured after the samples were processed via ultrafiltration, centrifugation or evaporation with redissolution in water. The pH values of processed and unprocessed urine and bile samples were measured in a similar manner. All the pH measurements were done at least in duplicates.

2.4. Preparation of buffers

2.4.1. Ammonium acetate buffer

Ammonium acetate buffer was prepared by dissolving ammonium acetate in deionized water to yield final concentration of 20 mM, and adjusting the pH to 5.1 with acetic acid.

2.4.2. Citrate buffers

Citrate buffer at different concentrations and pH were prepared by mixing appropriate amount of trisodium citrate salt and citric acid. Fine adjustment of the pH was made either with the citrate salt or the citric acid solution.

2.4.3. Phosphate buffers

Phosphate buffer was prepared by mixing appropriate amounts of NaH_2PO_4/Na_2HPO_4 salts. Fine adjustment of the pH was made either with the phosphoric acid or sodium hydroxide solution.

2.5. Standard samples of *I, II, III*

Stock solutions of **I**, **II**, **III** were prepared in water. The stability of **I** and **II** in plasma under various conditions and in phosphate buffer was monitored by spiking appropriate amount of stock solutions to obtain 0.01 volume of the final incubation mixture, followed by incubation at different periods of time and temperatures. Analytical standard curves of **II**, and **III** were prepared in buffered plasma for the LC/MS/MS analysis.

2.6. HPLC analysis of *I*

Analyses of samples of **I** in plasma and phosphate buffer at different pH were carried out on a Waters 2690 Alliance HPLC (Waters, Milford, MA) equipped with a photodiode array detector. The samples were injected from a refrigerated autosampler onto a Luna C_{18} column (2.0×150 mm, 3 μ) equipped with guard column (Luna C_{18} column (2.0×30 mm, 3 μ). The mobile phase consisted of Solvent A (ammonium acetate (pH 5.1; 20 mM)–acetonitrile (95:5, v/v)) and Solvent B (ammonium acetate (pH 5.1; 20 mM)–acetonitrile (5:95, v/v)) at a flow rate of 0.2 ml/min. A 20 min gradient was used with isocratic elution at 100% Solvent A for the first 4 min, followed by a 0.1 min linear gradient to 70% Solvent A/30% Solvent B, and then a 4.9 min linear gradient to 10% Solvent A/90% Solvent B, followed by 4 min isocratic elution with 10% Solvent A/90% Solvent B. The mobile phase was then returned to 100% solvent A and the column was reequilibrated for 6.9 min at the initial condition.

2.7. HPLC and LC/MS/MS analysis of *II* and *III*

For the analysis of the plasma samples of **II** and **III**, the Waters 2690 alliance HPLC was interfaced to the LCQ ion trap (Thermo Finnigan, San Jose, CA) mass spectrometer. For detection and quantitation, LC/MS/MS positive electrospray ionization mode was used. **III** (synthetic impurity and in vitro and in vivo hydrolytic product of **II**) undergoes conversions in the ion source of the mass spectrometer and forms $[\mathbf{II}+\mathrm{H}]^+$. Therefore, to selectively quantitate **II** by LC/MS, HPLC separation of **II** from potential interference of **III** was essential. As can be seen from their structures, these compounds are highly hydrophilic. Consequently, an HPLC mobile phase with a shallow gradient containing high percentage of water was used to resolve the two compounds using C18 reverse phase columns. The samples were then injected from refrigerated autosampler onto a Luna C_{18} column (2.0×150 mm, 3 μ) equipped with guard column (Luna C_{18} column (2.0×30 mm, 3 μ). The mobile phase contains a gradient system consisting of Solvent A (ammonium acetate (pH 5.1; 20 mM)–acetonitrile (95:5, v/v)) and Solvent B (ammonium acetate (pH 5.1; 20 mM)–acetonitrile (5:95, v/v)) at a flow rate of 0.2 ml/min. A 25 min gradient was used in which Solvent B with 2% initial condition was linearly increased to 17% in 10 min and then increased to 100% in 1.0 min, kept at 17% for 2 min and linearly decreased to 2% in 1.0 min, with equilibration time at the initial conditions for 9 min.

Plasma sample preparation of **II** and **III** for LC/MS/MS analyses, involved methanol precipitation. The aqueous-methanolic supernatant was then evaporated to dryness under vacuum and redissolved in water for the HPLC injections.

The use of an HPLC mobile phase with high water content for the analysis of **II** compromised the sensitivity of the LC/MS/MS. This is because, electrospray ionization is suppressed in the presence of solvent containing a high percentage of water, as a result of high solvation energy that would make ion desorption more difficult [10]. This is particularly magnified with **II** and **III** as they are highly polar, charged molecules. To increase the sensitivity, acetonitrile containing 1% acetic acid was used as a post-column modifier. A flow rate of 0.3 ml/min of the post-column modifier was found to be optimum for this purpose, and led to an increase in the sensitivity of the MS/MS by roughly an order of magnitude. The inclusion of a post-column modifier also improved the stability of the ion signals. A divert valve was utilized to direct the combined flow to waste during the first few minutes of each HPLC analysis to get rid of most of the matrices of the plasma samples and also during equilibration time. This practice helped to maintain a clean ion source

to stay clean and stabilize ion signals over extended period of time.

3. Results and discussion

3.1. pH of ex vivo plasma samples

3.1.1. Shift in pH of ex vivo rat plasma from physiological pH

Once plasma was removed from the body, the plasma pH was observed to increase with time. As shown in Table 1, when rat plasma was incubated at 37 °C, the pH shifted from the physiological pH (7.4) and increased to values greater than 8 within few hours of incubation. It is interesting to note that the change in pH occurred to the same degree regardless of the anticoagulants, i.e. EDTA, heparin or 0.1 M citrate buffer used in preparing plasma from blood. The initial pH of freshly harvested rat plasma or serum was also affected by the method used to euthanize the animal. For example, when CO_2 asphyxiation was used, the initial pH was in the range of 7.2–7.4, slightly lower than the normal physiological pH. On the other hand, when isoflurane was used, the initial pH of the harvested plasma was above 7.4.

3.1.2. Effect of pH shift on the stability of pH sensitive compounds

The change in pH of plasma could have significant impact on both enzymatic and chemical processes. For example, if the degradation processes are base or acid catalyzed, a small change in pH can lead to a significant change in the rate of degradation. At pH 7.4, the hydrogen and hydroxyl ions concentrations are 40 and 250 nM, whereas at pH 8, the concentrations are 10 and 1000 nM, respectively. This 4-fold increase in the hydroxyl ion concentrations from pH 7.4 to 8.0 may lead to a significant increase (or decrease) in the rate of chemical degradation. For example, after incubation of **I** at 37 °C in phosphate buffer for 1 h, about 12% of the compound was degraded at pH 7.4 whereas 40% of the compound was lost at pH 8.0 as shown in Table 2. This clearly indicates that small changes in pH can lead to significant changes in the rate of degradation of pH labile compound. When **I** was similarly incubated for 1 h at 37 °C in rat plasma that was stored in a freezer for 1 week and then thawed, 60% was lost, indicating the rate of degradation in this plasma sample to be markedly higher than the rate of degradation in pH 7.4 buffer (Table 2). This was attributed to the observed increase in pH of the rat plasma to pH 8.3. This indicates that stability data obtained in plasma ex vivo without pH control may not reflect the true stability of the compound in plasma under physiological conditions of pH 7.4. It is, therefore, imperative to devise experimental methods that would stabilize the pH of ex vivo plasma. Various methods have been used to stabilize the pH around the physiological pH as discussed below.

Table 1
Change of pH in rat plasma with time (37 °C)

Time (h) of incubation	Anticoagulant		
	EDTA	Heparin	Citrate
0	7.63	7.63	7.61
1	7.75	7.72	7.76
2	7.79	7.80	7.84
3	7.89	7.93	7.94
6	7.91	8.02	8.17

pH of plasma obtained from rats anesthetized with isoflurane. Incubations were done in capped tubes in duplicates, and the tubes were opened only during the pH measurements.

3.1.3. Methods for stabilizing pH of ex vivo plasma at physiological pH

To stabilize the pH of plasma ex vivo at the physiological pH of 7.4, two different methods were investigated: (1) use of 5–10% CO_2 and (2) use of small amount of phosphate or citrate buffers. Only data from rat plasma will be discussed in here, although rat serum, human plasma, and human serum samples were found to yield similar results.

3.1.3.1. Stabilizing pH of plasma by using CO_2 chamber. To minimize the change in pH of plasma ex vivo due to loss of CO_2 and to stabilize the pH around 7.4, plasma samples were loosely capped and kept in a chamber filled with 5–10% CO_2 at 37 °C. A 1 h equilibration under 10% CO_2 atmo-

Table 2
Percent **I** remaining after incubation at 37 °C with rat plasma or 0.3 M phosphate buffer at different pH values

Time (h)	Phosphate buffer (0.3 M)			Untreated rat plasma
	pH 7.0	pH 7.4	pH 8.0	pH 8.3
0.5	ND[a]	93	77	66
1	ND	88	60	38
2	99	77	32	9
4	ND	60	12	ND

Final concentration of **I** in the incubation mixture was 50 μM and all incubations were done in duplicates.
[a] ND, not determined.

sphere was found to stabilize the pH of plasma samples in the range of 7.4–7.5 as shown in Table 3. The pH remained unchanged over a period of 20 h. In comparison, the pH values obtained from tightly capped samples stored in water bath at the same temperature but at ambient atmosphere showed consistent increases to higher values.

3.1.3.2. Stabilizing pH of plasma by using phosphate buffer. The $H_2PO_4^-/HPO_4^{2-}$ buffer system has a p*K*a value of 7.2 with good buffer capacity in the pH range of 6.2–8.2. To determine an optimal buffer condition for stabilization of the plasma pH at the physiological condition, phosphate buffers with different initial pH values (5–7.4) and concentrations in the range of 0.06–1 M were prepared and evaluated at various plasma/buffer ratios (v/v). The best conditions for maintaining the plasma pH at about 7.4 were phosphate buffer concentrations greater than 0.5 M, pH of 7.0, and plasma:buffer ratios of 20:1 or 10:1 (Table 3). Although there is a slight increase in pH during the first few hours, the pH did not increase as dramatically as in the untreated control plasma and stabilized in the range of 7.3–7.7 for 24 h.

3.1.3.3. Stabilizing pH of plasma by using citrate buffer. The citrate buffer system has a pK_{a_3} value of 6.39 with good buffering capacity in the pH range of 5.39–7.39. To explore experimental conditions that would stabilize the pH at physiological pH, studies were carried out at citrate buffer concentration range of 0.6–2.0 M, and with initial pH values of 6.0 and 7.0, and at plasma:buffer volume ratios of approximately 20:1, 30:1, 40:1, 60:1 and 100:1. Best results in stabilizing plasma pH were obtained with pH 6 citrate buffer at a concentration of 1.0 M and plasma to buffer

Table 3
Change of pH of ex vivo rat plasma under different conditions at 37 °C with time

Time (h)	Naive plasma[a]	10% CO_2[b]	Citrate buffer[c]	Phosphate buffer[d]
0	7.27	7.27	7.30	7.26
1	7.98	7.42	7.44	7.54
2	8.32	7.43	7.53	7.63
4	8.46	7.48	7.67	7.73
6	8.63	7.46	7.71	7.59
24	8.80	7.46	7.68	7.55

All the pH measurements were done in duplicates.
[a] pH of fresh rat plasma incubated at 37 °C. Plasma was obtained from rats subjected to CO_2 asphyxiation.
[b] pH of fresh rat plasma incubated in 10% CO_2 incubator at 37 °C.
[c] pH of fresh rat plasma treated with 0.025 volume of citrate buffer of 1.0 M initial concentrations and initial pH value of 6, incubated over water bath at 37 °C over time.
[d] pH of fresh rat plasma treated with 0.05 volume phosphate buffer (pH 7, 1 M, initial pH and concentration values) incubated over water bath at 37 °C over time.

volume ratio of 40:1. Similar results were obtained with pH 6 citrate buffer at a concentration of 2.0 M and plasma to buffer ratio of 100:1 (Table 3). As in the case with phosphate buffer, the addition of citrate buffer did not completely prevent the pH from increasing to slightly above physiological pH during the first few hours, but this change was less than that observed in untreated control plasma samples and no further pH increases were observed above pH 7.6 for about 24 h.

3.2. pH of rat plasma samples processed using various methods

3.2.1. Shift in pH of rat plasma samples after various sample preparation methods

The pH of plasma samples was found to shift towards alkaline conditions during sample processing, such as ultrafiltration, centrifugation and extraction. For example, the pH of fresh rat plasma increased from pH 7.6 to pH 7.8 after 20 min of centrifugation at $1000 \times g$, whereas the pH of the resulting protein free filtrate (PFF) increased to 8.5. On the other hand, when plasma was evaporated to dryness and the residue redissolved in water, the resulting solution was found to have a pH exceeding 9.0. However, when the residue obtained after evaporation of the aqueous-methanolic supernatant was redissolved in equal volume of water, the resulting solution was observed to exhibit a pH values of about 9.5, a significant shift from the physiological conditions. Needless to say that, if this increase in pH of plasma samples is not controlled, it can present significant problems for the preparation and analysis of samples containing pH-labile compounds during drug disposition studies as discussed below.

3.2.2. Effect of change of plasma pH during sample processing

During sample work-up for pharmacokinetic studies, plasma samples are often cleaned up by precipitating of the plasma proteins with 1 to 2 volume of organic solvents such as methanol. Frequently, to further increase the sensitivity of the analytical method, the corresponding organic-supernatant is evaporated to dryness and the residue resuspended in an appropriate solvent before analysis. However, the pH of untreated plasma may change during sample work up as discussed above, potentially leading to misleading results, as was the case during pharmacokinetic studies of **II** in rats.

The initial extractions of **II** and its hydrolytic product (**III**) from the plasma samples, involved precipitation of plasma proteins with equal volume of methanol followed by direct injection of the supernatant into the LC/MS/MS. However, when the aqueous-methanolic supernatant solution was injected into the HPLC, peak splitting was observed for both compounds with the major component of each compound eluting with the solvent front. When water alone was used as injection solvent instead, well-resolved single peaks corresponding to each compound were obtained. As a result, the aqueous-methanolic supernatant was evaporated to dryness and redissolved in water for the HPLC injections and LC/MS/MS analyses of the plasma samples of **II** and **III**. When **II** was spiked into phosphate buffer at pH 7.4 or into tissue homogenates prepared from gastro-intestinal and lung tissues, quantitative recovery was obtained, indicating its chemical stability under these conditions. However, when **II** was spiked into blank rat plasma samples, and analyzed by LC/MS/MS after sample clean up with protein precipitation and evaporation as described above, low and variable recovery (30 to 50%) was obtained. To understand the cause of low recovery of **II** from plasma samples, stability studies were carried out by spiking of **II** into PFF prepared from naive plasma and into 5% rat albumin in pH 7.4 phosphate buffer. The results showed no degradation of the compound in the presence of albumin prepared in phosphate buffered at pH 7.4, but variable results were observed with PFF. When the compound was spiked into PFF and directly injected into the HPLC with no further treatment, no appreciable degradation was observed. However, when the spiked PFF was evaporated to dryness and the residue redissolved in water and injected into the HPLC, significant degradation was observed. This degradation occurred both when PFF alone was evaporated to dryness as well as when methanol was added to

PFF and the mixture was evaporated to dryness, similar to what was observed with blank rat plasma. The degradation was not concentration-dependent over the concentration range studied (subnanomolar to about 150 μM). As discussed above, the pH of the plasma extract after evaporation of the supernatant and redissolution in water increases to a value as high as 9.5, suggesting the possibility of pH-dependent degradation of **II** during sample work up. Stability studies of **II** in phosphate buffer at pH 9.0, confirmed that this compound undergoes pH-dependent hydrolysis to **III** with a half-life at pH 9.0 of about 2.5 h.

The suggestion that ex vivo increases in the pH of plasma samples during storage and sample processing may result in pH-dependent hydrolysis of **II** to **III** raised concerns as to how the pharmacokinetic study samples should be processed. Therefore, in order to avoid any pH-dependent degradation of **II** in plasma samples during sample processing steps, various methods of stabilizing plasma pH were investigated as discussed below.

3.2.3. Methods for stabilizing pH of processed plasma samples

To prevent pH-dependent degradation of **II** due to increase in the pH of plasma samples during storage and processing, various experimental conditions were investigated. As shown in Table 4, the ex vivo plasma pH was maintained in the acidic range when the plasma samples were treated with phosphate buffer of sufficiently low initial pH and sufficient concentrations. It was thus possible to prevent ex vivo degradation of **II** with the addition of small amount of phosphate buffer (0.1 volume of 0.3 M phosphate buffer at pH 5) to the plasma sample prior to sample processing. As a result, 0.1 volume of phosphate buffer (0.3 M, pH 5) was spiked into plasma samples from all pharmacokinetic studies of **II** before sample processing to stabilize the pH and prevent pH-dependent ex vivo degradation of **II**. Under these conditions, results from the pharmacokinetic studies of **II** in rats after single 10 umol/kg intraarterial infusion doses showed the systemic exposure to the parent to be about twice the exposure to the hydrolytic metabolite. Had the pH not been controlled, ex vivo degradation of **II** to **III** would have yielded results indicating comparable exposures to both compounds, clearly leading to a wrong conclusion.

For the same buffer pH values and concentrations, addition of buffer to plasma before extraction or addition of buffer to the organic supernatant extract produces similar results. Therefore, one can use a buffered organic solvent to simplify sample preparation steps by using appropriate buffer/organic solvent mixture.

An alternative to the above procedure is to collect a fixed volume of blood into tubes containing an anticoagulant and a fixed volume of phosphate buffer. Table 5 shows the pH of rat plasma harvested from blood collected into phosphate buffer and the pH values observed after precipitation of plasma with methanol, followed by evaporation and redissolution in water. As

Table 4
pH of plasma samples (in the absence and presence of phosphate buffer) before and after treatment with methanol followed by evaporation and redissolving in equal amount of water

Conditions	Anticoagulant	Initial rat plasma pH	pH of evaporated and redissolved methanol plasma extract[a]
Untreated plasma	EDTA	7.74	9.47
	Heparin	7.72	9.30
	Citrate	7.81	9.43
Plasma treated with 0.1 volume of phosphate buffer (pH 5, 0.3 M)	EDTA	6.58	6.91
	Heparin	6.61	6.79
	Citrate	6.60	6.84

All pH measurements were done in duplicates.
[a] pH values obtained after evaporation of the methanolic supernatant and redissolving in water.

Table 5
pH of plasma harvested from blood treated with phosphate buffer (blood:phosphate 10:1, v/v)

Phosphate buffer		Plasma	
Concentration (M)	pH	Initial pH	Final pH[a]
0.3	5.00	7.10	7.23
0.6	6.85	6.85	6.95

All pH measurements were done in duplicates.
[a] pH obtained after precipitation with methanol, the aqueous- methanolic supernatant was evaporated and the residue redissolved in equal volume of water.

shown in the table, addition of 0.1 volume of phosphate buffer (0.3–0.6 M, pH 5) was sufficient to maintain the plasma pH in the desired range. The addition of buffer directly into blood is particularly important to avoid any degradation of pH labile compound in the blood itself after sample collection and during preparation of plasma from blood by centrifugation.

One can of course use citrate buffers of lower pH values similarly for storage of plasma samples containing base-labile compounds. In fact, the use of citrate buffer may confer additional advantage as it can also be used as anticoagulant thus allowing a single additive to function as both the anticoagulant and as pH stabilizer.

3.3. pH shift in ex vivo bile and urine samples

The pH of fresh urine can range from pH 4 to 8 or even higher, and depends on the diet, health status, and the nature of any administered xenobiotic materials such as drugs. This is illustrated in Table 6, for fresh urine collected from two overnight fasted rats. As shown in the table the pH values of the fresh urine from the two rats were 6.2 and 7.1, respectively. However, upon incubation at either 37 °C or at room temperature, the pH values of the urine samples increased, becoming more alkaline with time with pH value exceeding 8.5 by 24 h after collections. However, when aliquots of the urine samples were evaporated to dryness and redissolved in a volume of water equal to the initial volume of urine, the final pH was slightly less than that of the fresh urine samples. As described above, urine contains bicarbonate, phosphate and ammonium ions, the relative ratio of which usually determines the pH of the urine samples. In the above case, it appears that the initial increase in pH of urine upon storage at either room temperature or 37 °C was due to the loss of CO_2. However, when the sample was evaporated to dryness, it resulted in the loss of ammonia, leading to a shift of the equilibrium to lower pH values where monobasic phosphates are predominantly formed. Due to this potentially wide swings in urine pH, it is imperative that the pH of ex vivo urine be controlled to avoid any ex vivo degradation of pH-sensitive compounds.

Fresh rat bile samples are alkaline due to the presence of appreciable amounts of bicarbonate that maintain the pH of bile in the alkaline range, despite the presence of fatty acids, bile salts and other organic acids etc. As shown in Table 6, the pH of bile samples can increase during sample processing. Upon evaporation of the bile samples to dryness and redissolution in equal volume of water, the pH increased to values greater than 9.0. To avoid ex vivo degradation of pH-labile compounds and metabolites such as acylglucuronides

Table 6
pH values of rat bile and urine samples ex vivo under different conditions

Time (h)	Bile (37 °C)[a]	Bile (25 °C)[a]	Bile[b] (dried)	Urine (37 °C)[a]	Urine (25 °C)[a]	Urine[b] (dried)
0	8.24 (8.38)	8.25	9.26 (9.44)	7.08 (6.24)	7.07	6.64 (5.89)
4	8.86 (8.48)	8.72		7.39 (7.30)	7.44	
24	8.97 (8.47)	8.94		8.97 (8.91)	8.68	

All pH measurements were done in duplicates.
[a] pH of fresh urine and bile incubated at 37 and 25 °C and different time points (data in parenthesis are from the second rat).
[b] pH of fresh urine and bile after evaporation to dryness by speedvac and dissolving in equal amount of water (data in parenthesis are from the second rat).

during storage and sample processing, it is important to stabilize the pH bile samples just as with urine and plasma samples.

4. Conclusions

The forgoing discussions indicate that the pH values of plasma, urine and bile samples change upon standing and storage as well as during sample processing. The pH of an unprocessed and untreated plasma sample can increase to a value as high as 8.8 upon storage at room temperature or at 37 °C. During sample processing, the pH can increase to a value as high as 9.5. This may result in the degradation of drugs and drug metabolites after samples have been collected and during sample processing. As a result, analysis of plasma samples should include experimental procedures that stabilize the pH to either the physiological condition or to other desired values. The above results indicate that the pH of plasma samples can be stabilized in the narrow pH range of 7.4–7.5 by placing the samples in a 10% CO_2 atmosphere at 37 °C. Addition of citrate or phosphate buffers can also be used to stabilize the pH of plasma, urine or bile samples. Whereas processing of plasma samples under a 10% CO_2 atmosphere may be feasible for stability studies or protein binding studies, this approach presents some technical challenges, particularly for samples requiring extensive preparation or long term storage. In those cases, the use of citrate or phosphate buffers may be of more practical utility. Regardless of the method used for pH stabilization, maintaining a stable pH in plasma, urine and bile samples is essential when attempting to characterize compounds which are themselves pH sensitive or which generate pH-sensitive metabolites.

Acknowledgements

We would like to acknowledge Saeho Chong, Rajesh Krishna, Elora Gupta, Mark Fancher, David Wang Iverson, Jian Wang, William Humphrey, Gregory Bisacchi, Steven Seiler, and Douglas Greene for the important suggestions they made during the preparation of this manuscript.

References

[1] J.C.L. Shaw, J. Nutr. 119 (1989) 1789–1798.
[2] J.W. Paxton, J. Pharmacol. Methods 5 (1981) 203–213.
[3] C.Y. Yeung, F.T. Lee, H.N. Wong, Acta Paediatr. Jpn. 34 (1992) 23–27.
[4] O. Bros, S. Jacobsen, Br. J. Clin. Pharmacol. 20 (1985) 85–88.
[5] O. Bros, G. Sager, D. Sandnes, S. Jacobsen, Br. J. Clin. Pharmacol. 15 (1983) 393–397.
[6] J.A. Henry, S.N. Mitchell, Br.J. Clin. Pharmacol. 11 (1981) 119P–120P.
[7] A.S. Kearney, L.F. Crawford, S.C. Mehta, G.W. Radebaugh, Pharm. Res. 10 (1993) 1461–1465.
[8] S. Khan, D.S. Teitz, M. Jemal, Anal. Chem. 70 (1998) 1622–1628 (reference therein).
[9] W.F. Ganong, Review of Medical Physiology, Lange Medical Publications, California, 1975, pp. 522–545.
[10] A.P. Bruins, Trends Anal. Chem. 13 (1994) 81–90.