Loss of Porphyrins from Solution during Analysis: Effect of Sample pH and Matrix on Porphyrin Quantification in Urine by “High-Performance” Liquid Chromatography

Sherry L. Perkins and Pam M. Johnson

We report the effect of sample matrix and pH on quantification of porphyrins by HPLC with fluorimetric detection. For aqueous solutions of pH < 2.5, HPLC peak heights of the porphyrins increased with decreasing pH, reaching a plateau at pH < 1.0. This loss of porphyrins from solutions with pH > 1.0 appeared to be due to a combination of microprecipitation and aggregation effects. No such “pH effect” was observed for urine samples supplemented with mixed-porphyrin standards. Addition of trace amounts of albumin to aqueous solutions also decreased these pH-related losses. These findings suggest a porphyrin–protein interaction that prevents microprecipitation and aggregation processes. We conclude that standard solutions of porphyrins for HPLC analysis should be prepared in a urine matrix. If aqueous solutions are used, then the pH must be adjusted to < 1.0. Urine samples from normal individuals require only adjustment of pH to < 2 before analysis; however, porphyrinic urines requiring dilution should be prepared with porphyrin-free urine diluent.

Various “high-performance” liquid-chromatographic (HPLC) techniques for quantifying urinary porphyrins have been published in the last 10 years (e.g., 1–4). We recently developed a high-speed HPLC technique for urinary porphyrin analysis (5), in which urine is acidified to pH 2.5–3.0, filtered, and injected directly into the HPLC. However, we encountered inconsistent results with this technique when urine samples from porphyrinic patients were serially diluted with hydrochloric acid before quantification.

Critical alterations in sample pH or matrix as a result of dilution may affect porphyrin solubility. Aqueous solubility of the porphyrins is determined by the acid/base characteristics of three reactive centers of the porphyrin molecule: the pyrrole =NH, the carboxylic acid (–COOH) side chains, and the pyrroline =N. The pyrrole =NH is an extremely weak acid, with a pH of approximately 16 (5), whereas the pk_a values for the various carboxylic acid groups have been reported as 3.5–4.5 (7) and 4.8–7.3 (6). Of the three reactive centers, the carboxyl groups and pyrroline nitrogen appear to be instrumental in determining the solubility of porphyrins in aqueous solution. Burnham (7) noted that, because ionized porphyrins are soluble in aqueous solution and uncharged porphyrins are soluble in organic solvents, solubility in organic solvents would be optimal between pH 2 and 5. In this pH range, most of the carboxyl groups are not ionized, and the pyrroline nitrogen is not protonated. These acidic conditions also enhance spontaneous oxidation of porphyrinogens to porphyrins. Most published extraction techniques (8, 9) exploit this “uncharged-pH region,” and many authors have reported very narrow pH ranges for efficient extraction. Although “tight” pH ranges have been advocated for organic extraction techniques, the published techniques for aqueous-sample preparation in HPLC analyses have included “looser” adjustment of pH to < 2 (10), “about 2” (11), or 2–6 (12), as well as direct injection of unpreserved urine (4) or of urine collected in sodium carbonate (13). Of these approaches, one would expect maximum aqueous solubility when the porphyrins are in the dicationic form (pH < 2) or carboxylate form (pH greater than ~ 8).

Additionally, the fluorescence intensity and the emission or excitation spectra of porphyrins are also determined by their ionization state (5). Alterations in the sample pH or matrix may therefore directly affect the fluorescent signal detected.

Many of the published HPLC methods do not adequately consider the complex porphyrin-ionization states outlined above. Here we report the results of our systematic investigation of the effect of pH in the range 0.5–2.5 with regard to porphyrin: fluorescence emission and excitation peak maxima; fluorescence intensity changes with protonation; precipitation or aggregation; oxidation/reduction; and solubility effects attributable to the matrix or salt formation. We also discuss optimal conditions for the quantification of porphyrins in urine by HPLC.

Materials and Methods

Reagents. All porphyrin standard solutions and other reagents were identical to those described previously (5). Additionally, hydrochloric salts of individual porphyrin carboxylic acids were obtained from Porphyrin Products, Logan, UT 84321.

Chromatographic conditions. Chromatographic equipment and conditions are described in detail elsewhere (5). In brief, the system consisted of a Perkin-Elmer Series 4 liquid chromatograph (Perkin-Elmer (Canada) Ltd., Montreal, Quebec, Canada) fitted with a 20-µL loop injector and a Perkin-Elmer LS-4 flowthrough fluorescence spectrophotometer (excitation wavelength 365 nm, emission wavelength 624 nm). Results were reported as relative fluorescence, i.e., individual peak heights expressed as a percentage of the integer for maximum peak height observed for all samples run on that day. The analytical column was a 3 cm × 0.43 cm (i.d.) cartridge column containing octadecylsilica particles 3 µm in diameter (Perkin-Elmer). Porphyrins were eluted with a linear gradient of 0.1 mol/L sodium phosphate buffer (pH 3.5) and methanol at a flow rate of 2.5 mL/min. The proportion of methanol was changed from 500 mL/L at injection to 950 mL/L in 2 min, held there for 2 min, then returned to initial conditions in a 1-min linear gradient. Re-equilibration time between injections was 3 min. The method response varies linearly with porphyrin concentration over the range 25–300 nmol/L. The within-day CV of the assay at 100 nmol/L was ±8% for all porphyrins.

Sample preparation. Mixed porphyrin standard stock solutions were prepared by dissolving the contents of the

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chromatographic marker standard (Porphyrin Products) in 150 μL of 6 mol/L HCl, then adding 9.85 mL of water. These commercial standards include a dicarboxylic acid porphyrin (mesoporphyrin), whereas urine contains no dicarboxylic-acid porphyrins. Working solutions containing each porphyrin at 100 nmol/L were prepared by volumetric dilution of the stock solution with dilute HCl or NaOH to yield solutions with pH values of 0.5–2.5.

Filtered samples were prepared by filtration through a 0.45-μm (pore size) Millipore filter attached to a 1-mL disposable syringe (Becton Dickinson Canada Inc., Mississauga, Canada). Unfiltered samples were mixed well, sampled immediately, and injected directly into the HPLC. Percentage losses of porphyrins during filtration were calculated as ([unfiltered – filtered]/unfiltered) × 100.

Porphyrin-free urine was prepared by acidification of normal urine to pH 4–5, followed by filtration through 0.45-μm (pore size) nylon filters (Millipore). Removal of the porphyrins was confirmed by HPLC with fluorescence detection.

To adjust the ionic strength of the solutions at pH 2.0 to that of the solution at pH 0.5, we added 1.0 mL of a 30.6 mol/L solution of KCl to 9.0 mL of water that had been adjusted to pH 2.0.

**Spectroscopy.** Absorption spectra were obtained with a Model DU-7 spectrophotometer (Beckman Instruments, Brea, CA 92621). Fluorescence spectra were obtained by introducing the sample directly into the cell of the Perkin-Elmer LS-4 fluorimeter, uncoupled from the HPLC. Emission spectra were obtained with an excitation wavelength of 400 nm. Excitation spectra were generated with an emission wavelength of 624 nm. Arbitrary amounts of each porphyrin carboxylic acid (HCl salt) were dissolved in 6 mol/L HCl, diluted 50-fold with water and then 10-fold with dilute HCl of various concentrations to give a range of pH values between 0.5 and 2.0 and absorbance values of <2.

**Results and Discussion**

Figure 1 illustrates the effect of sample pH on fluorescence peak height after HPLC separation of filtered porphyrin-standard solutions. The peak height increased for all porphyrins with decreasing pH. For the porphyrins found in urine, this effect appeared to plateau at pH <1.0.

**Effect of pH on Fluorescence**

Porphyrin fluorescence intensity, as well as emission and excitation peak maxima and minima, is affected by pH (6, 14). Polo et al. (15) recently studied the factors influencing fluorescence spectra of free porphyrins and found that the wavelength maxima of the three main emission peaks vary in the pH range 1–11, and that ionic strength strongly affects fluorescence intensity. They did not speculate as to whether this resulted from solubility, oxidation/reduction, or ionization effects.

To investigate whether the results summarized in Figure 1 were ascribable to spectroscopic effects, we obtained the fluorescence spectra of solutions of individual porphyrins in dilute HCl. In the pH range 0.5–2.0, shifts in emission and excitation wavelength maxima were only minimal; fluorescence intensity decreased with increasing pH, but no new bands were observed. These spectroscopic effects cannot, however, account for the results shown in Figure 1, because collection of the column eluate demonstrated that injection pH had no effect on the relative pH at the time of elution or on fluorescence detection of the porphyrins in our HPLC method.

**Effect of pH on Ionization and Solubility**

Falk (6) noted the pKa's of the porphyrin carboxylic groups are modified by the electrostatic effect of other dissociated groups on the same molecule. Thus the mean pKa's of mono-, di-, tetra-, and octacarboxylic acid porphyrins are 4.8, 5.7, 6.5, and 7.3, respectively. The pKa for the protonation of one of the pyrroline nitrogens is 5.5 (6), whereas the di-cation arising from protonation of both pyrroline nitrogens has a pKa of 1–2 (7). The visible absorption spectra of these various neutral, di-cationic, and di-anionic species are distinct and characteristic (6). Spectroscopic examination of solutions of individual porphyrins at various pH values <2 (data not shown) indicated all porphyrins were in the di-cationic form, suggesting maximum solubility in aqueous solution. However, as Figure 1 indicates, bringing the sample pH to <2, below the reported pKa of either the carboxyl or pyrroline group, was not sufficient to ensure 100% detection of all porphyrins in an aqueous sample.

**Precipitation and Aggregation**

Considerable confusion exists in the literature regarding the solubility of urinary porphyrins at acidic pH. Hill and
Bailey (12) reported that substantial losses of coproporphyrin from urine are caused by co-precipitation with other materials in slightly acidic conditions. In contrast, Rossi and Curnow (16) suggested that urine must be acidified to dissolve any precipitated calcium salts, which can adsorb porphyrins. In our study, no gross precipitate was visible in any of the solutions of pH 0·5–2·5. To investigate whether losses of porphyrins from solution of pH <2·5 were due to microprecipitation, we prepared mixed porphyrin standard solutions at various pH values, filtered (0·45 μm pore size) and unfiltered, and injected samples of each into the HPLC. For solutions of pH 0·45, <5% of the porphyrins are lost on the filters (Table 1). In contrast, 24·1–54·1% of porphyrins was retained for solutions of pH 2·24. Although these results may demonstrate a pH-dependent interaction of filter and porphyrin, our use of inert-fluorocarbon filters makes it more reasonable to assume that the losses occur through microprecipitation. The apparent microprecipitation with subsequent loss on the filter does not completely account for the differences observed between solutions of pH ≤1 and those of pH 2, because substantial decreases in fluorescence are also seen for unfiltered solutions (Table 2). The effects observed in the unfiltered samples were less severe than those observed in the filtered solutions, suggesting that, although some porphyrins are lost during the filtration process, filtrable non-fluorescent porphyrin complexes may also be present in solutions of pH 1–2 that are not solubilized in the methanolic mobile phase.

In addition to macro- or microprecipitation, the analytical recovery of porphyrins from solution may also be decreased by aggregation and (or) dimerization. Brown et al. (17) noted that protoporphyrin and coproporphyrin aggregate in aqueous solution, resulting in a decrease of the absorptivity and a broadening of the Soret band. In dilute solutions, electrostatic interaction and stacking of parallel overlapping porphyrin rings results in dimerization, which is both pH- and temperature-dependent. Redmond et al. (18) noted that aggregation of porphyrins in solution inhibits excitation to the triplet state and subsequently decreases the "fluorescent activity." They were able to monomerize several species of porphyrins with detergents and organic solvents (methanol or acetone). If porphyrin dimerization in solutions of pH <2 is contributing to the effect shown in Figure 1, one would anticipate that the high methanol content of the HPLC mobile phase would also monomerize these species, unless porphyrin mixing with the mobile phase is not sufficient to promote disaggregation. The effect of pre-injection mixing of samples with methanol was evaluated by diluting mixed porphyrin standard solutions of pH 0·5, 1·5, and 2·0 with an equal volume either of water adjusted to equivalent pH or methanol. Figure 2 illustrates that, except for mesoporphyrin, direct mixing with methanol did indeed restore the fluorescence intensity of the filtered solutions at pH 1·5 and 2·0 to values equivalent to or greater than that at pH 0·5. A slight pH effect was still apparent, however.

### Table 1. Effect of pH on Losses of Individual Porphyrins (100 nmol/L) during Filtration

<table>
<thead>
<tr>
<th>pH</th>
<th>Uro</th>
<th>Hepta</th>
<th>Hexa</th>
<th>Penta</th>
<th>Copro</th>
</tr>
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<tbody>
<tr>
<td>2.24</td>
<td>24.1</td>
<td>28.5</td>
<td>29.3</td>
<td>35.4</td>
<td>54.1</td>
</tr>
<tr>
<td>0.45</td>
<td>0.74</td>
<td>0.45</td>
<td>1.8</td>
<td>2.0</td>
<td>3.6</td>
</tr>
</tbody>
</table>

For clinical analyses, dilution of samples with methanol before analysis is not practicable. Methyl esters of the porphyrins are rapidly formed on standing in acidic methanol, resulting in double peaks (carboxylic acids + methyl esters) in HPLC analysis. Furthermore, dilution of samples eliminates the major advantage of the originally published method: minimal sample preparation and the ability to detect low concentrations of porphyrins in urine samples. Any dilution exceeding 10% may prevent detection of some porphyrins at the upper limit of normal in urine. Concentration of samples would eliminate the simplicity and rapid turn around time of the method.

### Matrix Effects

Westerlund et al. (19) have suggested that poor analytical recovery and precision of porphyrin analyses may result from matrix effects and (or) incomplete oxidation of porphyrinogens to porphyrins in the urine samples before analysis. The latter proposal does not account for the effect illustrated in Figure 1, because pH-dependent variability was observed for porphyrin standards that contained no porphyrinogens. Matrix effects due to differences in ionic strength have recently been shown to produce a "pH effect" on fluorescence (15). We used solutions of KCl to ensure that all aqueous samples attained equivalent ionic strength, but this did not eliminate the pH-dependent effects over the pH range 0·5–2·0. However, as shown in Figure 3, when undiluted porphyrin-free urine at various pH values was used instead of pH-adjusted water in the

### Table 2. Effect of pH on Relative Fluorescence of Unfiltered Solutions of HPLC-Separated Mixed-Porphyrin Standards

<table>
<thead>
<tr>
<th>pH</th>
<th>Uro</th>
<th>Hepta</th>
<th>Hexa</th>
<th>Penta</th>
<th>Copro</th>
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<tbody>
<tr>
<td>0.09</td>
<td>50</td>
<td>68</td>
<td>81</td>
<td>91</td>
<td>91</td>
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<td>0.31</td>
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<tr>
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<td>91</td>
<td>91</td>
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<tr>
<td>1.29</td>
<td>48</td>
<td>62</td>
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<td>1.44</td>
<td>47</td>
<td>61</td>
<td>69</td>
<td>89</td>
<td>91</td>
</tr>
<tr>
<td>1.88</td>
<td>32</td>
<td>45</td>
<td>58</td>
<td>78</td>
<td>91</td>
</tr>
<tr>
<td>2.22</td>
<td>24</td>
<td>34</td>
<td>42</td>
<td>55</td>
<td>65</td>
</tr>
</tbody>
</table>

Each porphyrin 100 nmol/L, in a mixture of standards.
implementable in aqueous solution. Thus, aqueous standards must have their pH adjusted to <1.0 or should be prepared in a porphyrin-free urine matrix. For urine samples with porphyrins <300 nmol/L, quantitative adjustment of pH to <2, filtration, and direct injection into the HPLC is satisfactory. Laboratories quantifying porphyrins by HPLC should be aware of these effects, especially when the patients' responses to treatment are monitored for porphyrin excretion by quantification of serial urine specimens.

![Figure 3](image-url)  
**Fig. 3.** Effect of pH on relative fluorescence intensity of HPLC-separated urine samples supplemented with porphyrins, 100 nmol/L each. Each point represents the mean (± SD) of triplicate determinations.

Formulation of mixed porphyrin standards, only mesoporphyrin showed a "pH effect." Except for albumin, physiological concentrations of some of the individual urinary components (Table 3) failed to prevent the "pH effect" in the presence of albumin concentrations of 30 mg/L. ~80% of added porphyrins was accounted for, compared with ~50% when water was the solvent. We cannot define the nature of the urine or albumin matrix effects, but we speculate that porphyrin/protein associations prevent or inhibit microprecipitation/dimerization processes.

![Table 3](table-url)  
**Table 3. Analytical Recovery of Porphyrins (100 nmol/L Each) Added to Aqueous Solutions Enriched with Various Urinary Components**

<table>
<thead>
<tr>
<th>Solution</th>
<th>Uro</th>
<th>Hepta</th>
<th>Hexa</th>
<th>Penta</th>
<th>Copro</th>
</tr>
</thead>
<tbody>
<tr>
<td>Albin, 300 mg/L</td>
<td>73</td>
<td>87</td>
<td>85</td>
<td>87</td>
<td>86</td>
</tr>
<tr>
<td>30 mg/L</td>
<td>73</td>
<td>84</td>
<td>84</td>
<td>87</td>
<td>86</td>
</tr>
<tr>
<td>3 mg/L</td>
<td>57</td>
<td>68</td>
<td>76</td>
<td>76</td>
<td>80</td>
</tr>
<tr>
<td>0.3 mg/L</td>
<td>57</td>
<td>70</td>
<td>69</td>
<td>68</td>
<td>78</td>
</tr>
<tr>
<td>Water</td>
<td>45</td>
<td>51</td>
<td>52</td>
<td>52</td>
<td>50</td>
</tr>
<tr>
<td>Creatinine, 16 mmol/L</td>
<td>57</td>
<td>65</td>
<td>67</td>
<td>67</td>
<td>67</td>
</tr>
<tr>
<td>Urea, 300 mmol/L</td>
<td>44</td>
<td>54</td>
<td>54</td>
<td>56</td>
<td>55</td>
</tr>
<tr>
<td>Ca²⁺, 8 mmol/L CaCl₂</td>
<td>64</td>
<td>68</td>
<td>71</td>
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<td>71</td>
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<tr>
<td>Mg²⁺, 8 mmol/L MgCl₂</td>
<td>67</td>
<td>71</td>
<td>73</td>
<td>74</td>
<td>74</td>
</tr>
<tr>
<td>Zn²⁺, 1.3 mmol/L ZnSO₄</td>
<td>60</td>
<td>66</td>
<td>67</td>
<td>57</td>
<td>67</td>
</tr>
<tr>
<td>Na⁺, 220 mmol/L NaCl</td>
<td>62</td>
<td>58</td>
<td>60</td>
<td>59</td>
<td>55</td>
</tr>
<tr>
<td>K⁺, 20 mmol/L KCl</td>
<td>47</td>
<td>47</td>
<td>45</td>
<td>47</td>
<td>42</td>
</tr>
</tbody>
</table>

* Relative to unsupplemented urine at pH 2.

References
18. Redmond RW, Land Ed, Truscott TG. Aggregation effects on...

Evaluation of the Biotinylated (Blugene™) vs 32P-Labeled cDNA Probes of β-Glucocerebrosidase: Relative Sensitivities in Genomic and Other Systems

Paula Strasberg

The sensitivity, rapidity, and ease of use of biotinylated (Blugene™, Bethesda Research Laboratories) and 32P cDNA probes have been compared, the probe being the cDNA for β-glucocerebrosidase (EC 3.1.2.45). With the Blugene kit I could detect 2 pg of biotinylated DNA on dot blots. However, under conditions of hybridization, the lower limit of detection for unlabeled cDNA (transblotted onto nitrocellulose) by its labeled counterpart was 5000-fold smaller (10 pg vs 50 ng) for the isotopically labeled probe. 32P- and Blugene-probes hybridized detectably with 0.5 and 10 μg, respectively, of transblotted EcoR I-digested genomic DNA, making the radioactive method 20 times as sensitive. However, color development was complete within 30 min to 3 h, whereas radiography required 12 h to one week. Blugene was also safer to use, and effective under appropriate conditions. The 32P method is expensive, hazardous, time-consuming, and technically difficult. This nonspecific procedure represents a desirable improvement in biotechnology.

32P-labeled DNA probes have been the sole reliable and suitably sensitive means for detecting gene sequences in Southern blots of genomic DNA. Within the last one to two years, however, a commercial system known as Blugene™ [Bethesda Research Laboratories (BRL), Burlington, Ontario, Canada] has been developed.1 DNA is biotinylated with the BRL nick translation kit; detection requires reaction with a simple streptavidin–biotin–alkaline phosphatase (EC 3.1.3.1) conjugate. The manufacturer claims that detection limits for this system are (a) as low as 0.25 pg of target DNA on Southern blots, and (b) 2 to 10 μg of restriction-digested human genomic DNA in single-copy genes on Southern blots.

To demonstrate a, investigators (1–3) digested, with EcoR I, samples of a plasmid containing a 1.1-kb Mst II fragment of the human β-globin gene in the EcoR I site of the plasmid pBR322, electrophoresed these digests on agarose gels, and transferred the resulting components to nitrocellulose by Southern blotting. The blots were then incubated with biotinylated samples of this same plasmid that had been labeled by nick translation with biotin-11-dUTP. To demonstrate b, they probed the Southern blot of EcoR I-digested human genomic DNA with the biotinylated 1.1-kb Mst II genomic fragment excised from the plasmid (1–3).

The avoidance of expensive, hazardous, short-lived isotopes is obviously appealing. Thus, in our laboratory, we have tried to replace our method of 32P labeling with this commercially available procedure. To compare the two methods, I used a cDNA probe, a method commonly used for detecting gene sequences in Southern blots of digested genomic DNA, for β-glucocerebrosidase (EC 3.1.2.45). Deficiency of this enzyme results in Gaucher’s disease, a lysosomal storage disease. I labeled the cDNA probe for β-glucocerebrosidase with [α32P]dATP, using the “random primed” DNA labeling method (Boehringer Mannheim), and separately with biotin-7-dATP, by the nick translation method as outlined by BRL (1).

Here I compare the 32P procedure with the biotin–streptavidin–alkaline phosphatase method for the detection of (a) the β-glucocerebrosidase gene in genomic EcoR I digests on Southern blots and (b) unlabeled probe, electrophoresed and transblotted onto nitrocellulose. Labeling the cDNA probe via the radioactive method proved to be the more sensitive, but nonradioactive labeling was much simpler and gave results more quickly.

Materials and Methods

Materials

SDS and nitrocellulose (Schleicher & Schuell, pure, cat. no. 40-00860) were from Bio-Rad (Mississauga, Ontario). The Blugene system, hybridization bags, nick translation system (cat. no. 81605B), regular and low-melting-point agarose (ultrapure, electrophoresis grade), 1-kb DNA ladder, phenol (ultrapure), and cesium chloride were from BRL. EcoR I, 500 units (1 unit represents the hydrolysis of 1 μg of DNA per hour) per 5 μL, and the random-primed DNA labeling kit (cat. no. 1004760) were purchased from Boehringer Mannheim (Dorval, Quebec). Formamid and glycine, “AnalaR” grade, were from BDH (Toronto, Ontario), and chloroform from Caledon (Georgetown, Ontario). 8-Hydroxyquinoline was from Fisher Scientific (Toronto, Ontario). "High salt buffer C" (per liter: 25 mmol of Tris HCl, 100 mmol of NaCl, 10 mmol of MgCl2, 100 mg of bovine serum albumin, and 2 mmol of β-mercaptoethanol, pH 7.5) was from International Biotechnologies, Inc. (New

1 Nonstandard abbreviations: BRL, Bethesda Research Laboratories; SDS, sodium dodecyl sulfate; SSC buffer, saline–sodium citrate; and EcoR I and Mst II, restriction enzymes.

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