LC Troubleshooting

What's Wrong with Ethylparaben?

John W. Dolan

Two problems submitted this month have one thing in common — unsatisfactory results when ethylparaben was used as an internal standard.

Internal standards are commonly used in liquid chromatography (LC) methods to help reduce errors in sample preparation and injection. If sample preparation and injection are precise, an internal standard can actually reduce the precision of the assay because it requires that two peaks be measured accurately, thus compounding the likelihood of measurement errors (an external standard requires the measurement of the height or area of only one peak).

In the present two cases, the internal standard peak is abnormal, whereas the remaining peaks in the chromatogram behave normally. The fact that both methods use an internal standard of ethylparaben, however, is coincidental — it is likely that similar problems could have occurred for any peak in the chromatogram. Both the internal standard and ethylparaben are red herrings here.

PEAK SHAPE PROBLEM

Q: For at least a year, I have been using a published method to analyze a proprietary steroid mixture. The isocratic separation uses 25% acetonitrile and 75% 50 mM phosphate buffer at pH 5.2. A C18 column is used at a flow rate of 2 mL/min with UV detection at 210 nm. The injection volume is 20 µL. The first two columns worked fine (Figures 1a and 1b), with well-shaped peaks and easy quantitation. Recently, I bought two replacement columns, and the peak shape is terrible for the ethylparaben internal standard (Figure 1c). I tried equilibrating the column for as long as 2 h to no avail. Washing the column with acetonitrile to remove late-eluted compounds did no good either. I spoke with the column manufacturer, who told me that the new columns are from a different lot than the old ones, but no differences in the chemistry of the packing material are known to exist. I even returned one of the new columns to the manufacturer, and it passed their new column test. What can I do to correct this problem? I have gone back to using one of the old columns, but I don't know what I'll do when it fails.

JWD: First, let me praise the reader for ordering and checking a new column before the old one had failed completely. Such forethought gives her some time to work on a solution before she can no longer perform her work. Too many analysts wait until the old column fails to see if the method will transfer to a new column. I spoke to the reader about possible changes in the system, and I am convinced that the only difference is the column. Further evidence is given by the differences in retention time between the two good columns under the same conditions (compare Figures 1a and 1b). Surprisingly, the peak-tailing problem (Figure 1c) is associated with ethylparaben. Parabens are often used as examples in manufacturers' application notes because they show off columns under the best conditions. To have a problem with a paraben and not the steroid is unusual.

One part of the method, however, should send up a red flag — the mobile-phase pH. Note that the pH is 5.2 with a phosphate buffer. Phosphate is not doing much in the way of buffering at this pH. My rule of thumb is that a buffer is effective at ±1 pH unit from the p*K*. Phosphate therefore provides useful buffering at pH 1.1–3.1, 6.2–8.2, and 11.3–13.3. A pH of 5.2 is well outside the recommended range.

So how do we change the method to correct the problem? We could adjust the pH so that it is within the appropriate range for phosphate, but we don't know how pH affects the method. It is likely we would have a lot of work ahead of us with this route. If the problem were a buffering problem rather than a pH problem, we could change to a buffer that is effective at pH 5.2. I recommended that the chromatographer try using acetate or citrate to test this hypothesis.

When an acetate buffer was used, the chemist reported a dramatic improvement in peak shape for the ethylparaben, while retaining acceptable resolution between it and the steroid. The acetate has a stronger absorbance than phosphate at 210 nm, so the wavelength was adjusted to 225 nm, and the amount of the ethylparaben internal standard was adjusted so that the peak heights were acceptable. This was a fortunate solution to the problem — it could have required a complete rework of the method.

So why did the method work for a year or more, then cause problems when a new column was used? Unfortunately, too many methods operate on what I refer to as "the edge of a cliff." You are perfectly safe until you make a wrong step — it may be small but fatal. For many LC methods, the chemistry of the separation system is marginal, yet it works. If the temperature, humidity, sample matrix, reagent source, or column chemistry changes, the method no longer works. The column recommended for this assay was a traditional C18 column that was not specially treated to remove residual metals and other minor impurities. If these impurities are important in the separation, and they often are, a small lot-to-lot change can adversely affect the separation as it did in the present case.

The cure for this problem is to prevent it from happening in the first place. Check the method for ruggedness by making reasonable variations in the pH, organic composition, temperature, column batch, and other important parameters. Only then will you know which parameter is important and which is not. This

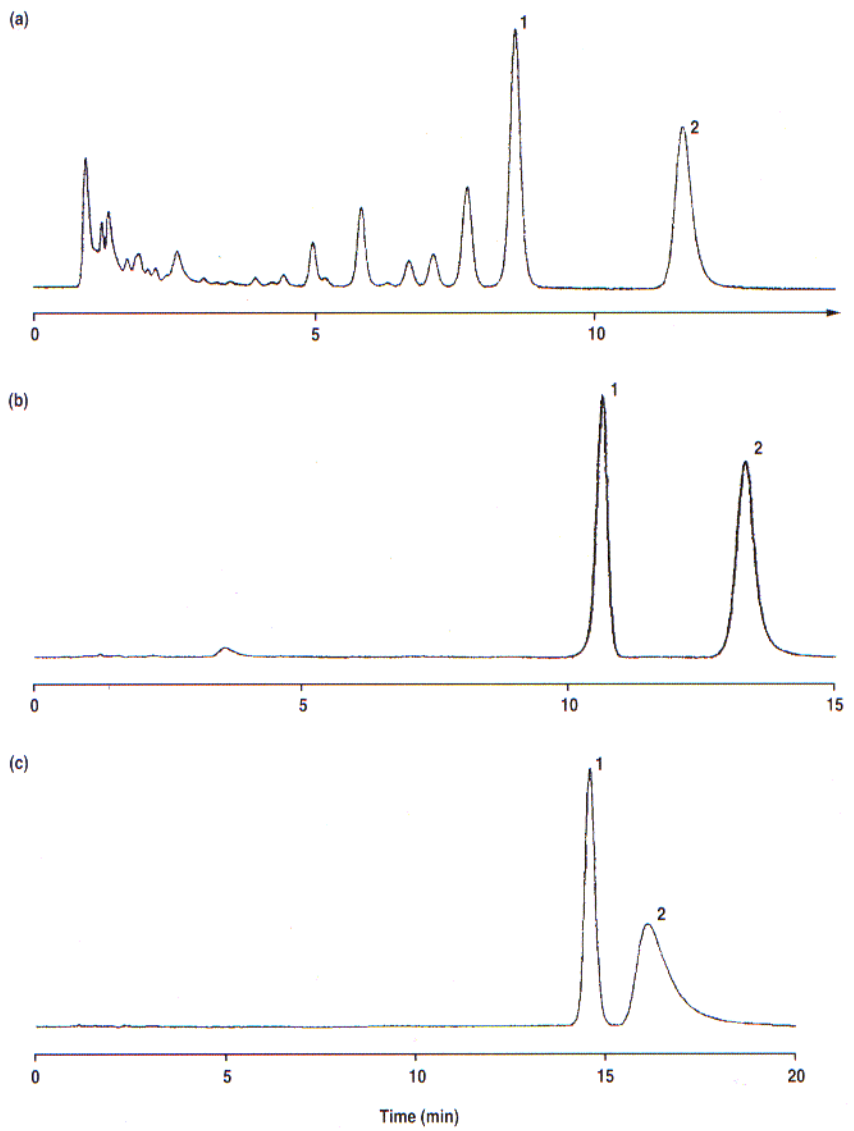

Figure 1: Separation of steroid sample with ethylparaben as the internal standard using (a) a good column, (b) a steroid standard with internal standard on a second good column, and (c) same as (b), but on a replacement column. Peaks: 1 = proprietary steroid, 2 = ethylparaben internal standard.

chemist was lucky — she tried out the new column before her old one was completely useless. Too many times the test is not made until the old column can no longer be used as a crutch to help you until you fix the problem.

SEPTUM MYSTERY

Q: For several years, I have been using a reversed-phase LC method to determine methylparaben and propylparaben content in our products. The method uses ethylparaben as an internal standard. The three-point calibration curve that we use contains three analyte concentrations and is linear. Recently, I started using a resealable septum to seal the calibration vials. During the validation process I observed unusual results. The ethylparaben seemed to have significantly less area when injected from a vial with a resealable septum than it did when a vial with a PTFE septum was used. This area difference was not observed with methylparaben or propylparaben; thus, the peak area ratio was drastically different between injections.

In summary, it appears that the resealable septum selectively absorbs ethylparaben, but does not affect methylparaben or propylparaben. I would greatly appreciate any suggestion that would help resolve this mystery.

JWD: This letter was accompanied by a sample of each septum. The PTFE septum is the standard white PTFE film septum used by many labs. The new septum appears to comprise a PTFE film laminated on a silicone rubber or similar material. Data from the initial experiments are shown in Table I, data set 1.

Table I: Septum Comparisons

	Standard Level	Septum Type*	Paraben Area		
			Methyl	Ethyl	Propyl
Set 1	1	R	7.4	27.7	29.8
		P	7.4	31.8	30.2
		P	7.4	31.9	30.1
	2	R	14.9	28.0	61.0
		P	14.8	31.5	60.9
		P	14.8	31.3	60.7
	3	R	22.3	27.5	91.6
		P	22.2	31.5	91.8
		P	22.2	31.3	91.5
Set 2	1	P	7.5	30.4	27.3
		P + R	7.7	28.0	29.1
		P + R	7.6	28.2	29.3
	2	P	15.1	30.8	58.0
		P + R	15.1	27.1	58.3
		P + R	15.1	28.0	58.1
	3	P	22.7	29.6	86.6
		P + R	22.8	27.2	86.6
		P + R	22.7	24.6	87.6

* R = resealable, P = PTFE, P + R = resealable with PTFE on both sides.

For the three standard levels, areas for the methyl- and propylparaben are consistent within a level and appear in the expected 1:2:3 ratio in standard levels 1, 2, and 3, respectively. The ethylparaben should be constant for all injections — and it is, but only within a single septum type. The resealable septum consistently produced areas that were ~12% smaller.

I talked to the chemist and asked the obvious questions. I found that the resealable septum was used with the PTFE side down but had been tried with the PTFE up with no apparent difference in response. The sample size was 10 µL. The retention time was ~8 min on a 25-cm C18 column. The vials were handled gently so that the liquid sample did not contact the septum.

I wondered whether the problem would be reduced if the septum were covered on both sides, so the experiments in Table I, data set 2, were run. In these experiments, a PTFE septum was placed on the vial next to the sample, then the resealable septum was added, with the PTFE side up, followed by the cap. The result was a "sandwich" of PTFE, silicone, and PTFE. The results look very similar to those of data set 1. The sample loss may be a little less pronounced, but it certainly is not eliminated.

CONCLUSION

So what's happening in these experiments? I'm afraid I don't have a good answer. I could justify a loss of methylparaben relative to the ethyl and propyl compounds. Volatility could account for such loss, with the lower molecular weight compounds being lost more quickly. I could also explain the selective loss of the less polar propylparaben as the result of some adsorptive interaction with the silicone septum; however, I cannot come up with a logical explanation for the selective loss of the ethylparaben relative to the more volatile methyl or more hydrophobic propyl analogs.

We use both types of septa in our laboratory and have encountered no similar problems. Perhaps one of you has experienced a similar phenomenon or can provide a logical explanation. Please write me c/o *LC•GC* or fax me at (503) 835-7930.

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