Selection, Evaluation, and Control of the Assay of the Pharmaceutical Product III

Statistical and Economic Evaluation of the Three-Component Infrared Spectrophotometric Assay of Aspirin Anhydride

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An experimental design of a series of three-component infrared spectrophotometric assays of various preparations of aspirin anhydride is statistically analyzed. Methods are shown for the proper choice of standards and the economical design of assays for a desired statistical confidence. Information as to stability and significance of physicochemical properties, such as melting point, is also obtained.

In previous papers of this series (1, 2), the statistical evaluation of final and bulk drug assays by ultraviolet spectrophotometry (1) and coulometry (2) was considered. The studies on aspirin anhydride (AA) as a possibly superior form for the oral administration of aspirin (3–6) necessitated the preparation of various batches. A three-component infrared spectrophotometric procedure was devised (4) to assay for the aspirin anhydride, aspirin, and salicylic acid in such material.

In general, three-component infrared spectrophotometric assays against synthetic standards are not as precise as the previous types of instrumental analysis discussed. Questions that should be answered concern the precision of such assays, the choice of the proper standard, the economics of replication within and among days of assays.

An experimental design to permit such conclusions is considered in this paper. The validity and reliability of the melting point as an indicator of purity is also considered. The weight loss and initial purity of the preparations are considered in light of the tendencies of the preparations toward instability. The statistical methods used are as discussed in standard texts (7).

EXPERIMENTAL

Four separate lots, A, B, C, and D, of aspirin anhydride which had been stored since preparation were used. They were sampled from the center of 100-Gm. amounts which had been sealed in brown glass bottles. The age of these lots and the analytical data obtained at the time they were originally prepared are given in Table I.

An additional lot, E, in use in Lab A as the assay standard, was included in the study. Ten samples, two of each lot, were submitted to Lab A on each of 5 days with requests for aspirin, salicylic acid, and aspirin anhydride assays of each. The coding of the samples was such (see Table II) that the operator was unaware of the duplication or that the same sample was submitted on sequential days. On each of the days of assay, aspirin anhydride, lot E, aspirin, and salicylic acid were run to serve as standards for the three-component assays on that day. The method of infrared assay was as given in the literature (4). The results of these assays are given in Table II.

Simultaneously with these studies, the lots were submitted on various days for weight loss on drying and melting point. These data are given in Tables III and IV.

RESULTS AND DISCUSSION

The analyses of variance of the anhydride, aspirin, and salicylic acid assays in AA are given in Tables V, VI, and VII. With all three, the assay variation among days exceeds the error among duplicate assays on the same day. Thus, assay duplication on any given day against the same standards may not be warranted if any obtained assay value is to be considered as characteristic of a particular preparation when assayed on any day.

The estimated standard deviations of a single infrared assay, including the error, day by lot interaction, and variation among days are ±0.9% for aspirin anhydride, ±0.4% for aspirin, and ±0.2% for salicylic acid for 87–100% pure anhydride. These standard deviations are given in per cent of the total sample, not in per cent of the component. The 95% confidence limits may be estimated by doubling these values.

Inspection of the data in Table II in comparison with Table I clearly shows that lot A would serve as a better standard than lot E.

In addition, appended to Tables V, VI, and VII are estimates of the components of variance for the data calculated from the standards run on the same day and for the average of the standards over the 5 days. In the cases of salicylic acid and aspirin assays of the AA, it apparently makes no difference whether a separate daily set of standards or the averages are used. However, the variability among days of assays for AA assay vanishes when the average of the standards is used and the total error among assays lessens. Thus, it is recommended that the same absorptivity constants be used in the calculation of all assays at all times. A set of

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TABLE I.—DATA ON ASPIRIN ANHYDRIDE (AA) LOTS AT TIME OF PREPARATION

Lot No.	A		B		C		D	
Age, mo.	12.0		9.2		8.0		7.5	
M.p., ° C.	85–86.5		77–81		81–83		82–84.5	
Lab	A	B	A	B	A	B	A	B
AA, %	101.0	100.3	96.0	96.3	97.5	99.8	99.1	98.7
Aspirin, %	0.2	0.1	3.0	1.8	2.1	1.0	1.0	0.8
Salicylic acid, %	0.1	0.1	0.3	0.6	0.0	0.2	0.4	0.1

TABLE II.—INFRARED ASSAYS OF ASPIRIN ANHYDRIDE (AA), ASPIRIN, AND SALICYLIC ACID IN 5 LOTS OF AA REPLICATED TWICE DAILY FOR 5 DAYS[a]

Day Submitted	Lot A Code No.	AA, %	Asp., %	Sal., %	Total, %	Lot B Code No.	AA, %	Asp., %	Sal., %	Total, %	Lot C Code No.	AA, %	Asp., %	Sal., %	Total, %
1	X110	99.3	0.7	0.0	100.0	X123	95.0	3.5	0.3	98.8	X131	87.0	9.0	0.4	96.4
	X110	99.0	0.7	0.0	99.7	X120	94.6	3.5	0.0	98.1	X130	87.3	9.1	0.1	96.5
2	X259	99.9	0.4	0.0	100.3	X215	96.4	2.8	0.0	99.2	X221	88.0	8.7	0.2	96.9
	X250	100.0	0.6	0.0	100.6	X210	97.2	2.4	0.0	99.6	X220	87.6	9.0	0.0	96.6
3	X353	100.8	0.3	0.5	101.6	X338	95.7	2.5	0.5	98.7	X317	87.7	9.1	0.6	97.4
	X360	100.1	0.1	0.5	100.7	X370	94.7	2.8	0.3	97.8	X340	87.7	8.9	0.6	97.2
4	X442	99.6	0.5	0.2	100.3	X453	94.4	2.8	0.0	97.2	X435	88.1	8.9	0.7	97.7
	X430	99.9	0.3	0.2	100.4	X460	94.4	2.8	0.0	97.2	X420	88.5	8.7	0.6	97.8
5	X537	99.5	0.3	0.0	99.8	X553	93.4	2.8	0.1	96.3	X549	86.2	9.0	0.3	95.5
	X520	98.7	0.4	0.0	99.1	X560	94.5	2.7	0.2	97.4	X580	86.4	9.5	0.3	96.2
Av.	...	99.7	0.43	0.14	100.3	...	95.0	2.86	0.14	98.0	...	87.5	9.0	0.38	96.8

Day Submitted	Lot D Code No.	AA, %	Asp., %	Sal., %	Total, %	Lot E Code No.	AA, %	Asp., %	Sal., %	Total, %
1	X145	96.7	2.8	0.1	99.6	X157	98.1	1.9	0.2	100.2
	X140	96.9	2.2	0.4	99.5	X150	98.1	1.9	0.2	100.2
2	X232	98.2	1.3	0.0	99.5	X241	98.1	1.2	0.0	99.3
	X230	99.1	1.5	0.0	100.6	X240	99.6	0.7	0.0	100.3
3	X326	97.0	1.4	0.4	98.8	X348	99.3	0.8	0.4	100.5
	X380	98.3	1.5	0.4	100.2	X320	99.2	0.8	0.4	100.4
4	X419	97.5	1.3	0.0	98.8	X422	99.6	1.1	0.3	101.0
	X400	98.2	1.2	0.1	99.5	X410	99.6	0.3	0.1	100.0
5	X527	96.6	1.6	0.0	98.2	X512	97.9	0.7	0.2	98.8
	X540	96.3	1.5	0.0	97.8	X530	97.7	0.9	0.1	98.7
Av.	...	97.5	1.63	0.14	99.3	...	98.7	1.03	0.19	99.4

[a] Calculated on the basis of the standards for that particular day.

TABLE III.—WEIGHT LOSS OF ASPIRIN ANHYDRIDE LOTS ON DRYING

Lot	Day Completed[a]	Weight Loss, %
A	8	0.06
	9	0.03
	10	0.04
B	8	0.13
	8	0.22
	10	0.13
C	8	0.24
	10	0.21
	10	0.27
D	9	0.08
	8	0.13
	10	0.20
E	6	0.08
	10	0.02
	10	0.04

[a] The three samples from each lot were respectively submitted on days 3, 5, and 8. The drying was started one day before date of completion.

standards may be run on a given day just to check on being within the control range of the series of standards.

It is interesting to note that lot A has shown no significant degradation for 12 months since its preparation, whereas lots B, C, and D prepared since that time and initially of less purity, have shown some run-down. Lot C has degraded as much as 10%. This confirms the effect of impurities on stability as previously noted (4). Comparison of weight loss values in Table III with the infrared assay (Table II) clearly shows that weight loss, presumably moisture, correlates with instability, as expected from previous studies (4). Melting points correlate with purity as expected. The best preparation, lot A, (Table II) of 99.7% AA has the highest and most reproducible melting point range, 83.4–83.8°, whereas the worst, lot C, of 87.5% AA, has the lowest melting point and the widest range. An individual melting point determination is most valid in the lower limit and least valid in the upper limit (see Table IV).

The estimated component of variance for variation among days of AA assay is $\sigma_D^2 = 0.43$, and for the error among assays on the same day is $\sigma_E^2 = 0.204$. The estimate of variance, σ_{AV}^2 for the average of nk assays could be

$$\sigma_{AV}^2 = \sigma_E^2/nk + \sigma_D^2/k \qquad \text{(Eq. 1)}$$

where n is the number of assays based on one set of standards or run on a given day, and where k is the number of days of assay. The variation among days is assumed to be independent of n. Equation 1 excludes the day × lot variation.

TABLE IV.—MELTING POINTS OF ASPIRIN ANHYDRIDE LOTS

Lot	Day Completed[a]	Melting Point Results, °C.	
A	11	83.4 –83.7	83.5–83.7
		83.5 –83.7	
	11	83.2 –83.7	83.3–83.8
		83.2 –83.8	
		83.4 –83.85	
B	10	79.2 –80.0	79.2–79.9
		79.2 –79.7	
	11	79.25–81.2	79.4–81.5
		79.3 –81.7	
		79.65–81.7	
C	10	78.0 –78.9	78.1–78.9
		78.2 –78.8	
	12	77.3 –83.5	77.6–84.6
		77.8 –83.9	
		77.7 –82.5	
		77.5 –88.4	
D	10	82.0 –82.4	82.1–82.5
		82.2 –82.6	
	12	81.6 –83.1	81.6–82.9
		81.7 –82.7	
		81.4 –83	
E	10	82.0 –82.5	82.1–82.5
		82.0 –82.3	
		82.3 –82.6	
	12	82.2 –96	82.1–96.4
		81.9 –96.8	

[a] The two samples from each lot were respectively submitted on days 3 and 5, each to be run at least in duplicate.

TABLE V.—ANALYSIS OF VARIANCE OF THE INFRARED ASSAY FOR ASPIRIN ANHYDRIDE IN ASPIRIN ANHYDRIDE[a]

Sources of Variation	Sum of Squares	Degrees of Freedom	Mean Square[b]	Quantities Estimated by M.S.
Days (D)[c]	19.12	4	4.78	$10\,\sigma_D^2 + 2\,\sigma_{DL}^2 + \sigma_E^2$
Lots (L)	966.37	4	241.6	$10\,\sigma_L^2 + 2\,\sigma_{DL}^2 + \sigma_E^2$
Days × lots (D × L)[d]	8.38	16	0.524	$2\,\sigma_{DL}^2 + \sigma_E^2$
Error (E)[e]	5.10	25	0.204	σ_E^2
Total	998.96	49	20.39	

[a] Lots among days, 5; lots, 5; and replicates, 2. [b] F test: days × lots: error = 2.57; 5% F = 2.1 ∴ significant at 5% level. Lots: days × lots = 461; 0.1% F = 7.9 ∴ significant at ≪0.1% level. Days: days × lots = 9.13; 0.1% F = 7.9 ∴ significant at 0.1% level. [c] Estimated variance; $\sigma D^2 = 0.43$ ($\sigma D^2 = 0$ if av. of daily standard used). [d] Estimated variance; $\sigma DL^2 = 0.16$ ($\sigma DL^2 = 0.15$ if av. of daily standards used). [e] Estimated variance; $\sigma E^2 = 0.204$ ($\sigma E^2 = 0.212$ if av. of daily standards used). Estimate of variance of a single assay of a given lot = $\sigma^2 = \sigma D^2 + \sigma DL^2 + \sigma E^2 = 0.76$ ∴ $\sigma = 0.89\%$ ($\sigma = 0.60\%$ if av. of daily standards used).

Equation 1 may be combined with a cost function to determine the most economic distribution of assays among and within days. A possible cost function could be

$$C = nkc_1 + kc_2 \qquad \text{(Eq. 2)}$$

where C is the total cost, c_1 is the cost of a replicate assay per day, and c_2 is the cost of setting up the assay on any given day. In general c_2 far exceeds c_1, but for $k = 1$, σ_{AV}^2 must always exceed σ_D^2, no matter the number of replicates.

TABLE VI.—ANALYSIS OF VARIANCE OF THE INFRARED ASSAY FOR ASPIRIN IN ASPIRIN ANHYDRIDE[a]

Sources of Variation	Sum of Squares	Degrees of Freedom	Mean Square[b]	Quantities Estimated by M.S.
Days (D)[c]	3.80	4	0.950	$10\,\sigma_D^2 + 2\,\sigma_{DL}^2 + \sigma_E^2$
Lots (L)	482.62	4	120.65	$10\,\sigma_L^2 + 2\,\sigma_{DL}^2 + \sigma_E^2$
Days × lots (D × L)[d]	1.747	16	0.1092	$2\,\sigma_{DL}^2 + \sigma_E^2$
Error (E)[e]	1.09	25	0.0436	σ_E^2
Total	489.25	49	9.985	

[a] Lots among days, 5; lots, 5; and replicates, 2. [b] F test: days × lots: error = 2.51; 5% F = 2.1 ∴ significant at 5% level. Lots: days × lots = 1.104; 0.1% F = 7.9 ∴ significant at ≪0.1% level. Days: days × lots = 8.70; 0.1% F = 7.9 ∴ significant at 0.1% level. [c] Estimated variance; $\sigma D^2 = 0.084$ ($\sigma D^2 = 0.075$ if av. of daily standards used). [d] Estimated variance; $\sigma DL^2 = 0.033$ ($\sigma DL^2 = 0.029$ if av. of daily standards used). [e] Estimated variance; $\sigma E^2 = 0.044$ ($\sigma E^2 = 0.040$ if av. of daily standards used). Estimate of variance of a single assay of a given lot = $\sigma^2 = \sigma D^2 + \sigma DL^2 + \sigma E^2 = 0.131$; $\sigma = 0.36\%$ ($\sigma = 0.38\%$ if av. of daily standards used).

TABLE VII.—ANALYSIS OF VARIANCE OF THE INFRARED ASSAY FOR SALICYLIC ACID IN ASPIRIN ANHYDRIDE[a]

Sources of Variation	Sum of Squares	Degrees of Freedom	Mean Square[b]	Quantities Estimated by M.S
Days (D)[c]	1.077	4	0.2692	$10\,\sigma_D^2 + 2\,\sigma_{DL}^2 + \sigma_E^2$
Lots (L)	0.433	4	0.1082	$10\,\sigma_L^2 + 2\,\sigma_{DL}^2 + \sigma_E^2$
Days × lots (D × L)[d]	0.3852	16	0.0241	$2\,\sigma_{DL}^2 + \sigma_E^2$
Error (E)[e]	0.2150	25	0.0086	σ_E^2
Total	2.1100	49	0.0431	

[a] Lots among days, 5; lots, 5 and replicates, 2. [b] F test: days × lots: error = 2.80; 5% F = 2.1 ∴ significant at 5% level. Lots: days × lots = 4.50; 5% F = 3.0 ∴ significant at 5% level. Days: days × lots = 11.2; 0.1% F = 7.9 ∴ significant at 0.1% level. [c] Estimated variance; $\sigma D^2 = 0.0245$ ($\sigma D^2 = 0.046$ if av. of daily standards used) [d] Estimated variance; $\sigma DL^2 = 0.0078$ ($\sigma DL^2 = 0.011$ if av. of daily standards used). [e] Estimated variance; $\sigma E^2 = 0.0086$ ($\sigma E^2 = 0.0078$ if av. of daily standards used). Estimate of variance of a single assay of a given lot = $\sigma^2 = \sigma D^2 + \sigma DL^2 + \sigma E^2 = 0.0409$ ∴ $\sigma = 0.204$ ($\sigma = 0.254$ if av. of daily standards used).

As was developed in an analogous situation (8), the condition,

$$n = \sqrt{c_2/c_1}\,(\sigma_E/\sigma_D) \qquad \text{(Eq. 3)}$$

specifies the number of replicates per day for the minimum standard error, σ_{AV}, of the assay at a specified total cost C, where the value of n from Eq. 3 substituted into a rearranged Eq. 2 determines the number of days, k, of n assays.

The condition of Eq. 3 also specifies the value of n for a specifically desired σ_{AV} at minimum cost where this value of n substituted into a rearranged Eq. 1 determines the number of days, k, of n assays.

CONCLUSIONS

The reliability of the infrared assay for aspirin anhydride, salicylic acid, and aspirin is obtained from the studies, and statistical evaluations are

given. The estimated standard deviations of a single infrared assay of aspirin anhydride are AA, ±0.9%; aspirin ±0.4%; and salicylic acid, ±0.2%; in terms of per cent of the total sample. The 95% confidence limits may be estimated by doubling these values. This is based on a new set of standards run on each day. This information demonstrates the foolishness of reporting apparent values by tenths of per cent. For example, if the obtained numerical value of salicylic acid and aspirin in one assay ranges from 0.0 to 0.4%, the amounts present are actually immeasurable and may be considered absent by the sensitivity of the assay.

The greatest assay variation is among days of assay rather than among replicates on a given day. If a routine control procedure is put into effect, assay duplication on the same day is an unnecessary expense. If the most precise assay estimate is desired at minimum cost, special consideration must be given to assaying the sample for several days.

The use of one set of standards or an average value for standards gives less total variability in the AA assay, ±0.6%, and no apparent variation among days. The variability in the aspirin and salicylic acid assays is not significantly changed by the use of either daily or averaged standards.

A comparative study such as this permits the choice of the better standard. For example, lot A should be preferred to lot E. Lot A, the oldest of the lots but of the highest initial purity, has shown no significant degradation for over a year's time. Less initial purity results in greater degradation. It is also shown that low per cent weight loss (considered as low moisture content) correlates with the higher stability.

The correlation of melting point with purity permits the establishment of a desired m.p. and expected error. The lower limit for good AA should be 81.8 ± 0.2°.

The insignificance of salicylic acid in these AA lots of varied purity show that little definition is lost on ignoring it. In fact, a statistical evaluation of data assayed by a two-component assay, for aspirin and AA only, gave less error of estimation.

REFERENCES

(1) Garrett, E. R., This Journal, **51**, 672(1962).
(2) Garrett, E. R., and Olson, E. C., *ibid.*, **51**, 764(1962).
(3) Garrett, E. R., *ibid.*, **48**, 676(1959).
(4) Garrett, E. R., Schumann, E. L., and Grostic, M. F., *ibid.*, **48**, 684(1959).
(5) Garrett, E. R., *J. Am. Chem. Soc.*, **82**, 711(1960).
(6) Gray, J., Jones, P., and Feenstra, E., *Toxicol. and Appl. Pharmacol.*, **2**, 514(1960).
(7)(*a*) Brownlee, K. A., "Industrial Experimentation," Chemical Publishing Co., Inc., Brooklyn, N. Y., 1953. (*b*) Bennett, C. A., and Franklin, N. L., "Statistical Analysis in Chemistry and the Chemical Industry," John Wiley & Sons, Inc., New York. N. Y., 1954. (*c*) Davies, O. L., "Statistical Methods in Research and Production," 3rd ed., Hafner Publishing Co., New York, N. Y., 1957.
(8) Garrett, E. R., and Savage, G. M., *Antibiotics & Chemotherapy*, **5**, 273(1955).

Antibacterial Activity of Mixtures of Quaternary Ammonium Compounds and Hexachlorophene

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Hexachlorophene and several quaternary ammonium compounds in admixture were evaluated for antibacterial activity by *in vitro* techniques commonly employed for the evaluation of lotions, creams, and ointments. It was observed that a maximum decrease in activity occurred as the components of the mixture approached equimolar ratios. The formation of a water-insoluble complex tends to diminish the antibacterial activity of mixtures when tested by broth dilution or agar plate techniques.

Numerous examples of the inactivation of bactericidal cationic substances may be found in the literature and several have been reviewed by Lawrence (1).

The anionic nature of hexachlorophene would also suggest a lesser antibacterial action in the presence of quaternary ammonium compounds. The problem of ascertaining the extent of inactivation, if any, was undertaken because of the possible usage of both hexachlorophene and quaternary ammonium compounds in items such as lotions, powders, and creams. The methods selected for bacteriological evaluation were those commonly associated with the *in vitro* evaluation

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