hydrophobic drugs such as phenobarbital and phenacetin dissolve faster in gastric juice than in 0.1 *N* HCl, mainly because of the lower surface tension of the former. The question arises whether there will be any difference between the dissolution rates in the two solvents if an HCl solut on of the same pH and surface tension as the diluted gastric juice is used. This question is important in relation to what kind of dissolution medium should be recommended for an *in vitro* dissolution test in the pharmacopeias.

The problem was studied with phenacetin as model substance. Two dilutions of gastric juice were made, one from samples of gastric juice with a relatively low surface tension (35–40 dynes cm.$^{-1}$) and one from samples with a relatively high surface tension (45–50 dynes cm.$^{-1}$). Rates of dissolution were determined using these two dilutions of gastric juice and two HCl solutions of the same pH and surface tension as the gastric juice mixtures. The surface tension of the HCl solutions was adjusted at the correct value by addition of polysorbate 80.

Figure 8 shows that phenacetin dissolves nearly at the same rate in hydrochloric acid as in gastric juice when the two media are adjusted at the same pH and surface tension. As far as this drug is concerned, an HCl solution adjusted to a surface tension of 40–50 dynes cm.$^{-1}$ is a better dissolution medium for an *in vitro* test than an HCl solution with no surfactant added. To see if this also applies to other drugs further studies are needed.

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Keyphrases

Dissolution kinetics—gastric fluid
Phenobarbital—dissolution rates
Phenacetin—dissolution rates
Gastric fluid, HCl solution, compared—drug dissolution
Surfactant concentration—dissolution effect
Surface tension—dissolution effect
UV spectrophotometry—analysis

Evaluation of the Effect of Isomerization on the Chemical and Biological Assay of Vitamin D

Analysis of Fat-Soluble Vitamins X

By J. A. KEVERLING BUISMAN, K. H. HANEWALD, F. J. MULDER, J. R. ROBORGH, and K. J. KEUNING

The reversible thermal isomerization of calciferols (D) to precalciferols(P), leading to a temperature-dependent equilibrium in solutions, interferes with many chemical and biological analytical procedures for the evaluation of vitamin D products. Two quantities should be distinguished: the potential vitamin D content (precalciferol + calciferol) and the actual vitamin D content (calciferol only). Of these two, only the potential vitamin D content is of importance to the buyer of vitamin D. Biological vitamin D assays give the potential vitamin D content, provided sample and reference standard dilutions are equilibrated by heating simultaneously to be sure that all have the same P–D ratio. This equilibration is superfluous in most of the chemical determinations of the potential vitamin D content. The actual vitamin D content can be determined by chemical, but not by biological, assay.

IT IS UNFORTUNATE that the assay of vitamin D preparations still frequently gives rise to difficulties. The reproducibility of biological assays is often poor, especially when assays from different laboratories are compared. The correlation of biological and chemical determinations is often unsatisfactory.

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A source of errors which has drawn too little attention is the reversible isomerization of the calciferols in solutions to the corresponding precalciferols, forming an equilibrium mixture (1–3).

In 1931 Reerink and Van Wijk (4) observed that the physical constants of freshly irradiated solutions of ergosterol changed spontaneously. Velluz *et al.* (3) isolated the 3,5-dinitrobenzoate of the responsible intermediate in 1949 and called it

precalciferol. Legrand and Mathieu (5) used infrared spectrophotometry to prove the mutual isomerization of the dinitrobenzoates of ergocalciferol (D_2) and preergocalciferol (P_2) leading to an equilibrium by first-order reactions. Verloop *et al.* (6, 7) and Velluz *et al.* (8, 9) established the structure of precalciferol. Havinga and coworkers (6, 10) investigated the chemical background of the isomerization of conjugated trienes and established that the isomerization rate is not affected by the solvent, acidity, ultraviolet light, catalysts, and free-radical reaction inhibitors.

Hanewald *et al.* (11) determined by ultraviolet absorptiometry the first-order rate constants of the isomerization of cholecalciferol, and their dependence on temperature. The isomerization rates of ergocalciferol and cholecalciferol are virtually equal (12). This should be a relief for many bioanalysts who have been compelled to standardize vitamin D_2 samples against cholecalciferol standards.

Shaw and Jefferies (13) reported an antirachitic activity of preergocalciferol of only 40% of the activity of ergocalciferol in rats; Bolliger (14) found 56% and Bekemeier and Pfordte (15) found 34%. For precholecalciferol Hanewald *et al.* (11) reported 35% of the activity of cholecalciferol in a chick test. The lower activity of the precalciferols must be due either to poor absorption or to lower intrinsic activity, the latter possibly originating exclusively from calciferols formed within the animal body by conversion of precalciferols (11, 13). A solution prepared from pure calciferol or pure precalciferol will after a time contain both compounds in a ratio dependent on storage time and temperature. This is also true for vitamin D preparations prepared from irradiation mixtures because during the photochemical manufacture of both calciferols, primarily the precalciferols are formed and the calciferols are formed from these by thermal isomerization. Whether the equilibrium will be reached clearly depends on the processing time and temperature, and if these are short or low, respectively, the ratio precalciferol/calciferol in the commercial product may be higher than conforms to equilibrium ratio at the ambient temperature and it will change until equilibrium has been reached.

These facts should be taken into account when assaying vitamin D or standardizing analytical methods.

THEORETICAL CONSIDERATIONS

Actual and Potential Vitamin D Content—When defining the vitamin D content there must be a distinction between the *actual vitamin D content*, *i.e.*, the content of calciferol alone, and the *potential vitamin D content*, meaning the sum of the contents of calciferol and precalciferol. Of these two, only the potential vitamin D content of a sample is independent of conditions during, preceding, and after the analysis and only the potential vitamin D content is a lasting characteristic and as such suitable for the evaluation of preparations. The actual vitamin D content concerns the sample at one distinct moment and it has a limited value, only in connection with the sample's antecedents.

By not distinguishing between potential and actual vitamin D contents a rather chaotic situation has arisen with regard to biological and chemical vitamin D assays, standardization, reference standards and their use, declared potencies, comparison of experimental results, stability tests, *etc.* Moreover, it must be emphasized that considerable errors in the actual vitamin D content may be introduced during analysis unless anything liable to change the ratio of both compounds, *e.g.*, heating, is carefully avoided (12).

Calculation of Equilibrium Compositions and Reaction Times—The isomerization constants for $P_3 \underset{k_2}{\overset{k_1}{\rightleftharpoons}} D_3$ are (11):

$$\log k_1 = \frac{-4200}{T} + 10.29$$

$$\log k_2 = \frac{-5180}{T} + 12.53 \quad (k \text{ in min.}^{-1};\ T \text{ in °K.})$$

At equilibrium the cholecalciferol fraction in % of the potential D content is:

$$f = \frac{100\ D_3}{P_3 + D_3} = \frac{100\ k_1}{k_1 + k_2}$$

The time needed to reach 0.99 × the equilibrium fraction of the formed component, starting from solutions of pure cholecalciferol or pure precholecalciferol, is

$$t_{0.99} = \frac{2 \times 2.303}{k_1 + k_2} \text{ min.}$$

Table I contains the fractions and the times calculated for a series of temperatures. These times vary from years at below 0°C. to some minutes at sterilization temperature (120°).

On the other hand, the time needed in practice to approach the equilibrium at a certain temperature, T may be reduced to a few days if one starts with a mixture equilibrated at some higher temperature T_0, as can be seen in Table I. This time is calculated from

$$t_{0.99} = \frac{2.303}{k_1 + k_2} \log 100 \left(1 - \frac{f_0}{f}\right) \text{ min.}$$

f_0 and f are the equilibrium fractions at the temperatures T_0 and T, respectively; k_1 and k_2 are the rate constants in min.$^{-1}$ at T.

Implications of the Distinction Between Potential and Actual D Content—The constancy of the potential D content and the inconstancy of the actual D content due to isomerization lead to a number of important consequences.

(*a*) Only the *potential* D content is useful for the evaluation of vitamin D preparations, because it is

TABLE I—CALCULATED EQUILIBRIUM FRACTIONS AND TIMES $t_{0.99}$ NEEDED TO REACH 0.99 × THE EQUILIBRIUM FRACTION OF THE FORMED COMPONENT (D_3 or P_3)

Temp. T_0, °C.	Equilibrium Fractions, % of P + D P_3	D_3	Pure P_3 or D_3 at T_0	Time $t_{0.99}$ Needed Starting from Equilibrium Mixture at T_0, Days at T 0°	20°	30°	40°	50°
−20	2	98	16 years	—				
0	4	96	350 days	—				
20	7	93	30 days	85	—	—	—	—
30	9	91	10 days	126	5	—	—	—
40	11	89	3.5 days	153	10	2.0	—	—
50	13	87	1.3 days	173	13	3.0	0.6	—
60	16	84	0.5 days	195	15	4.5	1.5	0.3
80	22	78	0.1 days	225	19	6.0	2.0	0.7
100	28	72	30 min.	250	21	7.0	2.5	0.8
120	35	65	7 min.	270	23	7.5	2.7	1.0

reproducible and independent of the antecedents of the sample.

(*b*) This applies still more to the comparison of experimental results obtained at different places and at different moments. Stability tests should never compare actual but always potential D contents since it cannot be decided whether a decreased actual D content is due to reversible isomerization or to irreversible decomposition.

(*c*) For the analysis of many formulations it is necessary to isolate or concentrate the vitamin D. This can hardly be done without saponification, extraction, and other operations involving an elevated temperature and consequently a change in P–D ratio. In such formulations the potential D can still be determined but the actual D cannot.

(*d*) It must be emphasized that knowledge of the actual D content can only be valuable if the circumstances preceding the analysis are well defined and if the potential D content is known.

(*e*) In a determination of the actual D content the circumstances starting from the moment of sampling should be such that isomerization virtually cannot occur, *viz.*, by handling at low temperature.

These points are illustrated by the following examples.

1. Preparations heated during manufacture (sterilization) and cooled abruptly have a low relative actual D content (D/D + P) which will be maintained by storing at 0° or below. If dosage is based on any actual D assay in the cooled product this will lead to overdosing.

2. Two solutions of pure crystalline calciferol in oil are prepared (*a*) by dissolving in ether, mixing with oil and evaporating the ether at 20° *in vacuo*, and (*b*) by dissolving in ethanol, mixing with oil, and evaporating the ethanol during 30 min. at 100°. The actual vitamin D content of both is determined and each is diluted with oil to make the actual D content 10 mg./ml. (solutions *a* and *b*). The fresh solution *a* contains no appreciable amount of P (0.01 mg./ml.); however, the fresh solution *b* approx. 4 mg. of P per ml.

After storage for 1 month at 20°, these solutions will show actual D contents of: (*a*) 9.3 mg./ml. and (*b*) 13.0 mg./ml. The potential D contents will be unchanged: 10.0 mg./ml. in *a* and 14.0 mg./ml. in *b*.

3. Animals having a body temperature of 40° are injected intramuscularly with dilutions of these fresh solutions *a* and *b*. Suppose half the depot has been absorbed a month after injection. The remaining half depot of *a* will contain at that moment 45% but *b* 65% of the calciferol present in the injected dose.

The conclusion of all this is that the potential D content is the value important for the consumer. Evaluation and assay of vitamin D preparations should be confined to the determination of the potential D content.

METHODS OF EVALUATION

When determining the *actual* vitamin D content (or P content), one should observe the following points. (*a*) The conditions (temperature and time) from the moment of sampling until measurement should be such that an appreciable conversion D ⇌ P does not occur, *e.g.*, <1 hr. at 40°, or <8 hr. at 20°, or <3 days at 0°, or 1 year at −20°. (*b*) D and P should be separated completely without conversion and then determined separately, or (*c*) D and P are not separated but freed from interfering contaminations and then one or both or the ratio are determined, and the sum of both (potential D).

An example of *b* is TLC followed by Nield's reaction. An example of *c* is chromatographic purification (*e.g.*, alkaline alumina), followed by a Nield determination of P + D and ultraviolet absorptiometry for the ratio. D might be determined in the presence of P by infrared absorptiometry at 900 $cm.^{-1}$ [exocyclic methylene band missing in P (5)]. Or P is converted by iodine into tachysterol of which the maleic anhydride adduct does not interfere with the Nield reaction of D (7).

The determination of the *potential* vitamin D content is not limited by the conditions mentioned above. There are three possibilities.

1. Purification without separating P and D and determination by a common reaction (*e.g.*, Nield reaction).

2. Separation and purification; the P and D fractions are analyzed separately and the results added, or the fractions are reunited and analyzed together (*e.g.* TLC, followed by extraction of the separate or combined P and D fractions, and Nield reaction).

3. Comparison of a measurable property of a (purified) sample solution and a potential vitamin D standard solution, after equilibration of both under rigorously identical circumstances (*e.g.*, ultraviolet absorptiometry or biological assay).

Effect of the Isomerization P ⇌ D on Biological Assays—The uncertainty as to the P–D ratio combined with a considerable difference between the biological activities of P and D interferes with the reliability of biological assays.

In biological assays the physiological effects of a sample and of a reference standard are compared and from this the content of the sample relative to that of the reference standard is calculated. A predominant condition for a realistic assay is the availability of a dependable reference standard.

A reference standard prepared by dissolving crystalline ergocalciferol or cholecalciferol will contain, after some time, a mixture of the original calciferol and, as a result of isomerization, precalciferol. The biological response to this standard will now represent the resultant of two compounds differing markedly in biopotency. A further complication is the new shift in isomerization that begins at the moment of administration and is caused by the animal body temperature. Even if only the actual D is biologically active the unknown degree of isomerization of the standard makes it unfit for use in the determination of the actual D (or the P) with a bioassay.

For the same reasons quantitatively equal physiological effects will not be obtained if sample and standard contain equal amounts of actual D but different amounts of P.

In other words an equal antirachitic effect does not prove the equality of the actual D contents of sample and standard unless the P–D ratios are equal.

The situation is quite different, however, in the case of the more important potential vitamin D content. In the first place the amount of potential vitamin D in a standard solution is exactly known, *viz.*, the amount of calciferol used in its preparation, provided the vitamin D has not been destroyed by irradiation or oxidation. In the second place dilutions of sample and standard, containing equal amounts of potential vitamin D (calciferol plus precalciferol) will cause quantitatively the same antirachitic effect, if beforehand any possibly different P–D ratios have been made equal by heating at the same temperature until the equilibrium pertaining to this temperature is reached.

In addition reference standard and sample should be applied in virtually identical formulations, *e.g.*, dissolved in a vegetable oil and both should be administered to the animals in the same way. Complications due to absorption differences of calciferols and precalciferols cannot occur if formulation, equilibration, and administration of all dilutions of sample and reference standard are identical.

Equilibration of Sample and Standard for Bioassay—The following example describes an equilibration of the dilutions of the sample and the reference standard for the bioassay of potential vitamin D in oil samples.

For dilution use a vegetable oil, pure and clear and free from peroxides, acids, and halogens.

Weigh sufficient quantities of sample and reference standard and dilute with vegetable oil and antioxidant solution to obtain all dilutions needed for administering, containing the required vitamin D concentrations and equal concentrations of antioxidant (*e.g.*, 0.02% of BHA). Mix each dilution thoroughly by swirling under nitrogen.

Heat all flasks simultaneously for 2.5 hr. at 80°C. in an oven. Cool simultaneously to room temperature. Proceed with the bioassay according to common practice (16–19).

The simultaneousness is essential and care must be taken to handle *all* dilutions of standard *and* sample in the same way with respect to temperature and time. When not in use all dilutions are stored in a refrigerator.

In addition to the precautions mentioned above to prevent oxidation and other irreversible conversions it is good practice to replace air by nitrogen each time after the flasks have been opened. In case the sample is not a solution in oil, some degree of isolation may be necessary to precede dilution by vegetable oil, *e.g.*, saponification, extraction, evaporation, dissolution. These operations will not alter the potential vitamin D content if they are carried out by reliable methods which prevent losses. The P–D ratio may change if heating is involved, but this will not influence the results because of the subsequent equilibration.

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Keyphrases

Vitamin D—analysis
Isomerization effect—vitamin D assay
Actual vitamin D content—calciferol concentration
Potential vitamin D content—calciferol and precalciferol concentration