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NEW CONCEPTS IN DENSITOMETRY

CONTROL ANALYSIS OF PHARMACEUTICAL FORMULATIONS

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SUMMARY

A study of a number of different pharmaceutical compounds has shown that with the calibration function $A^2 = m'c + n'$ (A = peak area, c = concentration) linear calibration curves can be obtained with concentrations between 1 and 10 μg per spot. For concentrations in the range of 0.3 to 1.0 μg the function $h = m''c + n''$ (h = peak height) is preferred at higher amplification. This actually means that with the densitometric evaluation of directly spotted liquid samples concentrations in the range 0.03–5 mg/ml can be analysed.

The time saved by direct sample spotting has been demonstrated with a typical example where nine analyses of a product with a 0.2 μg/ml concentration of active principle (2 μl/spot) can be carried out in one day, with a reproducibility of $\pm 1.4\%$ relative S.D. For the same samples, using classical elution procedures, 3–4 days are required and extraction of samples prior to spotting (with danger of decomposition) is necessary.

The analysis of thyroacetic acid, which appears as an enzymatic breakdown product of thyronine and has a $\lambda_{max.}$ value of 235 nm, has been carried out by UV reflectance at concentrations of 50 ng/spot using a standard addition approach. This is a good example of the applicability of this method to very low concentrations and at relatively short $\lambda_{max.}$.

Instrumental modifications which facilitate the measuring process and data handling are discussed.

INTRODUCTION

From the number of publications[1–12] that have recently appeared it seems that quantitative thin-layer chromatography (TLC) with *in situ* evaluation via densitometric methods has found a prominent place in pharmaceutical analysis. This technique has been used for routine analysis of both active principles and formulations. However, there is still a discrepancy in the accuracy of this method compared to TLC evaluation via elution and spectrophotometric methods. In recent publications[13,14] it has been shown that with the data-pair technique it is possible to carry out quantitative densitometric measurements by UV reflectance which are comparable with data obtained by

elution techniques. Similar observations were made with *in situ* fluorescence measurements[15,16]. The data-pair technique is based on an application of unknowns and standards in pairs according to a special scheme[14] and permits three analyses per plate.

The kinds of reproducibility that can be expected with this approach can be seen in Table I, which compiles the results for a number of practical applications in routine analysis.

TABLE I

SOME PRACTICAL APPLICATIONS OF THE DATA-PAIR TECHNIQUE IN ROUTINE ANALYSIS OF PHARMACEUTICALS

Mode of operation	*Active principles, dosage and preparations*	*Spotting conditions*	*Concentration per spot (μg)*	*Rel. S.D. (%)*
UV reflectance	Active principle X, 0.2 mg/ml, ampoules I	2 μl directly	0.4	±1.4
UV reflectance	Active principle X, 0.2 mg/ml, ampoules I	5 μl after extraction	4	±1.6
Fluorescence[15,16]	Active principle X, 0.2 mg/ml, ampoules I	10 μl directly	2	±1.2
UV reflectance	Active principle Y, 1 mg/ml, ampoules II	2 μl directly	2	±1.1
UV reflectance	Ergotamine tartrate	2 μl	5	±2.7
UV reflectance[17]	Caffeine, 40 mg Aminopyrine, 125 mg, Butalbital*, 45 mg dragée III	2 μl after extraction	2 6.2 2.2	±1.6 ±1.2 ±1.9
UV reflectance[17]	Caffeine, 25 mg Aminopyrine, 125 mg, Butalbital*, 50 mg dragée IV	2 μl after extraction	2 10 4	±1.9 ±1.8 ±1.9
Fluorescence	Digoxin	2 μl	0.2	±1.1

* 5-Allyl-5-isobutylbarbituric acid.

Equivalent accuracy of another technique does not justify its replacement of an established analytical method. Time and cost factors and simplicity are of high importance in laboratory routine. A major consideration in this regard is the possibility of spotting ampoule solutions and other liquid pharmaceutical formulations directly onto the plate without prior extraction and complex sample preparation. Elimination of the extraction step is not only time saving but it also reduces possible dangers of decomposition of sensitive compounds.

Such a direct sampling approach seems possible with densitometry, contrary to elution techniques, due to the higher sensitivity of this method. Also with the small volumes applied, no overloading of the chromatograms due to presence of buffers, glycerine or other excipients is usually encountered. On the other hand, a flexible densitometric evaluation system is needed to permit specific, sensitive, accurate and reproducible measurements in spite of markedly varying dosage concentrations. It was, therefore, the aim of this study to investigate different calibration functions as to their

suitability in the lower concentration ranges ($<1\ \mu g/\mu l$). Ergotamine tartrate was chosen as a model system and the mode of measurement was UV reflectance spectroscopy. The possibility of adapting standard addition techniques was studied with thyroacetic acid.

Again in the interest of time optimization, some instrumental modifications were also considered in order to facilitate data handling and measurement steps.

EXPERIMENTAL

All chromatographic developments have been carried out on commercially coated silica gel Merck F_{254} plates (Merck, Darmstadt, G.F.R.).

Spot application was made with Drummond disposable microcaps® (Drummond, Broomall, Pa., U.S.A.).

All solvents were of reagent grade. The following developing systems were used: for ergotamine tartrate, chloroform–ethanol (8:2); for active principle X in ampoules I, chloroform–ethanol–NH_3 (70:30:2); and for thyroacetic acid, benzene–acetic acid–dioxane (90:4:25). The plates were dried at room temperature.

Sandoz active principle X is a basic compound with $\lambda_{max.} = 320$ nm and active principle Y is a similar compound with $\lambda_{max.} = 280$ nm. No significant changes in measured reflectance have been noticed with any of the systems investigated after 1-h exposure of the plates to UV light.

Standard solutions for the determination of compound X in ampoules (80, 100 and 120% of expected value) have been prepared in a medium identical with the ampoule solution. After spotting, a mixture of chloroform–methanol (1:1) was applied over the same spot with the same microcap to ensure complete application of the test solutions.

Densitometric measurements were carried out with a Zeiss chromatogram spectrophotometer (Zeiss, Oberkochen, Württemberg, G.F.R.), an Infotronics Model CRS 104 integrator (Infotronics, Boulder, Colo., U.S.A.) and a W & W Model 2211 recorder (W & W electronic, Münchenstein, Switzerland).

RESULTS AND DISCUSSION

The results for a dilution series of ergotamine tartrate are shown in Fig. 1. The following calibration functions were tested: peak area A obtained by electronic peak integration ($A = mc + n$), peak area squared ($A^2 = m'c + n'$) and peak height ($h_{recorder} = m''c + n''$), all plotted as functions of concentration. The regression curves for these functions were drawn for different concentration regions and the corresponding correlation coefficients are shown in Table II. An underlined coefficient depicts the most ideal function for a particular concentration range, as represented in Fig. 1.

The linearity of the calibration curve is influenced by the properties of the test compound, such as absorption maxima, specific extinction and molecular weight, but also by chromatographic parameters (polarity and R_F values) and diffusion phenomena which tend to influence the amount of substance on the surface of the adsorbent layer. As it is this material on the surface which is mainly detected by reflectance measurements it is not surprising that one particular calibration function is only useful

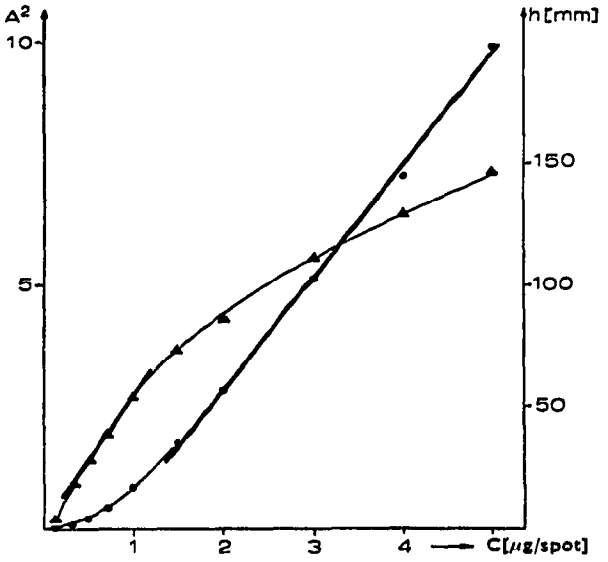


Fig. 1. Calibration plots for the functions $A^2 = m'c + n'$ (●—●) and $h = m''c + n''$ (▲—▲).

over a very limited concentration range, as can be clearly seen from Fig. 1 and Table II. The limitations of $A^2 = m'c + n'$, which is an approximation of the Kubelka–Munk function, have been discussed extensively elsewhere[18]. The results show that the latter calibration function can be quite useful in the concentration range above 1 µg/spot, whereas a better linearity is obtained with the function $h = m''c + n''$ at lower concentrations. Another advantage of this function is the relative ease of manual peak height measurements at lower signal-to-noise ratios (higher recorder amplification due to lower concentrations).

TABLE II

CORRELATION COEFFICIENTS FOR DIFFERENT CONCENTRATION RANGES

Range of concentration (n = number of data points)	*Correlation coefficients for*		
	$A = mc + n$	$A^2 = m'c + n'$	$h = m''c + n''$
0.1–5 µg/spot ($n = 10$)	0.9886	0.9936	0.9765
1.5–5 µg/spot ($n = 5$)	0.9958	0.9992	0.9951
0.3–1 µg/spot ($n = 4$)	0.9996	0.9886	0.9999

Concentrations below 1 µg/spot are quite common with direct analysis of ampoule solutions. A more extensive investigation of the concentration range 0.2–1 µg/spot, in conjunction with peak height functions, was therefore carried out. The results are shown in Fig. 2. The correlation is highly significant. The recommended spotting volumes and the corresponding calibration functions are given for several concentration ranges of the active principle in the original solution (Table III). This table has been compiled for ergotamine tartrate and would change somewhat for other groups of substances and chromatographic systems. But as a rule of thumb Table III

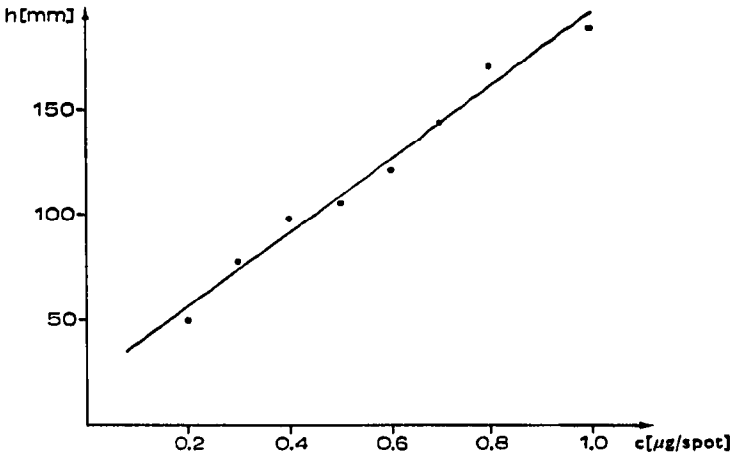


Fig. 2. Calibration curve for the low-concentration region (<1.0 µg) with eight data points.

has proven valid for a large number of other pharmaceutical compounds. As a result one can therefore directly analyse ampoules with concentrations of ~0.03–5 mg/ml without extraction or dilution procedures. The gain in analysis time can be demonstrated with the example presented in Table I (analysis of ampoules I, 0.2 mg/ml); with the direct spotting technique nine densitometric analyses were carried out in one day

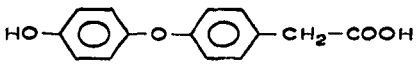
TABLE III

RECOMMENDED SPOTTING VOLUMES FOR CERTAIN CONCENTRATION RANGES AND CALIBRATION FUNCTIONS

Calibration function	*Spotting volumes (µl)*	*Concentration of active principle (mg/ml)*
$h = m''c + n''$	2	0.15–0.4
	5	0.06–0.16
	10	0.03–0.08
$A^2 = m'c + n'$	2	0.5 –5
	5	0.2 –2
	10	0.1 –1

with ±1.4% relative S.D. Three to four days were needed for nine analyses by extraction of the active principle, TLC, and elution of chromatographic zones. As already mentioned, the manual determination of peak height is preferred over an electronic approach, due to poor baseline and lower signal-to-noise ratio.

In cases where a 10% relative S.D. can still be tolerated it is possible to carry out UV reflectance measurements in the low nanogram region. This can be of interest for the determination of low levels of impurities or breakdown products. An example of this nature is thyroacetic acid —[*p*-(*p*-hydroxyphenoxy)phenyl]acetic acid[19]— which appears as an enzymatic breakdown product of thyronine. The $\lambda_{max.}$ is at 235 nm and the concentration region investigated was 30–600 ng. For the calibration func-



tion $h = m''c + n''$ correlation coefficients between 0.9950 and 0.9966 were observed. Some practical analyses were carried out by means of standard addition methods as demonstrated in Fig. 3. An equal amount of unknown was added to each concentration of the standard test solution and by extrapolation of the resulting calibration curves one can determine the concentration directly on the concentration ordinate

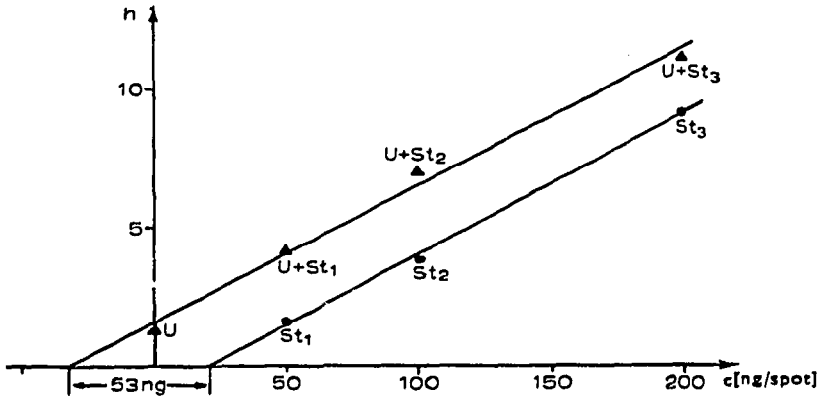
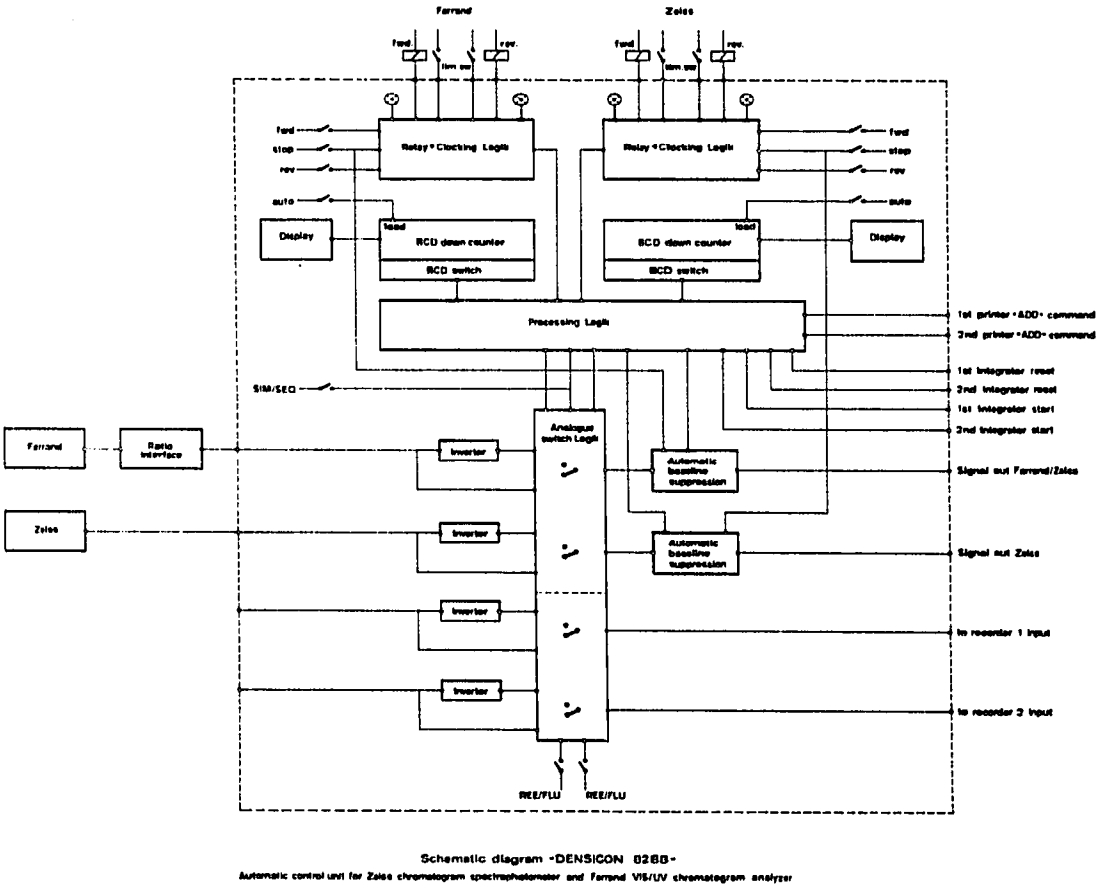


Fig. 3. Calibration plots and analysis for thyroacetic acid, using a standard addition approach. St = standard; U = unknown.

between the two intersection points. In the example shown, the deviation from the true value (50 ng) was 6%. It should also be noted that the measurements were carried out at a low UV wavelength ($\lambda_{max.} = 235$ nm), which comes close to the lowest technically feasible working wavelength of $\lambda = 220$ nm for reflectance measurements on silica gel. Similar concentrations can otherwise only be detected on TLC by fluorescence or radiochemical methods.

Instrumental modifications

In order to ensure a time-optimized operation of the available densitometers, we have adapted two instruments to a central integrator. They can be alternately and automatically switched through to the integrator. This mode of operation is more efficient. While one plate is being positioned and readied for measurement, the other is being scanned and peak areas integrated. Each instrument can be pre-set with regard to the operation mode, *i.e.* one instrument could for example work on the fluorescence mode while the other simultaneously operates in the UV or visible mode. A schematic diagram and a photograph of this arrangement are shown in Figs. 4 and 5. The number of scans (1–9) and the initial direction of scan can be pre-selected and are continuously indicated during operation. Off-line evaluation of the integrator values with a desk calculator is currently being investigated.



Schematic diagram "DENSICON 6288"

Automatic control unit for Zeiss chromatogram spectrophotometer and Ferrand VIS/UV chromatogram analyzer

Fig. 4. Schematic diagram for Densicon automatic control unit.

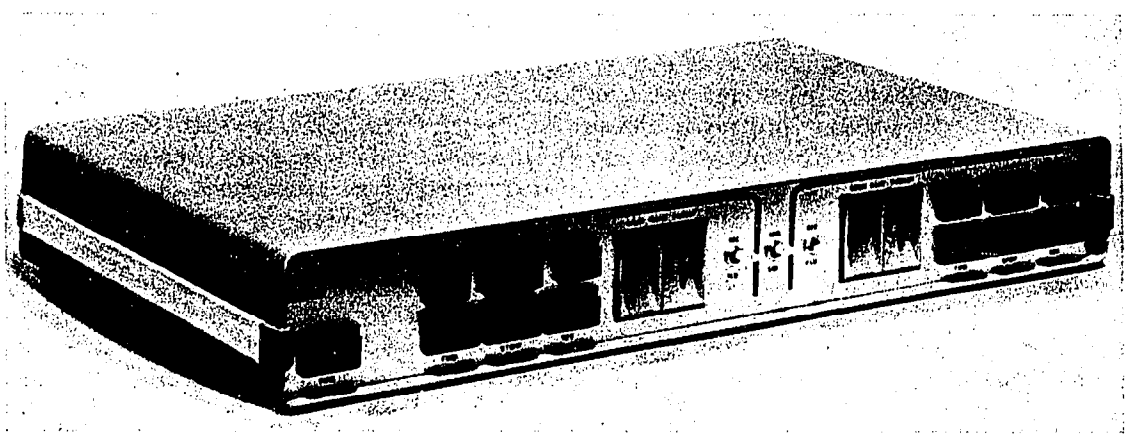


Fig. 5. Densicon automatic control unit.

CONCLUSIONS

It has been demonstrated in this study that densitometric techniques can be adapted to routine analysis of pharmaceuticals with considerable time saving and simplicity, without loosing any accuracy in comparison with classical methods. The potential for automating the data acquisition and processing steps is reasonably good.

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