Journal of Chromatography, 194 (1980) 217–223
Elsevier Scientific Publishing Company, Amsterdam — Printed in The Netherlands

CHROM. 12,732

Note

Sample-solvent-induced peak broadening in the reversed-phase high-performance liquid chromatography of Aspirin and related analgesics

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(First received November 13th, 1979; revised manuscript received January 30th, 1980)

Acetylsalicylic acid (Aspirin; 3) is a widely used analgesic, both alone and in combination. On exposure to moisture the drug undergoes hydrolysis to yield salicylic acid (4), the content of which must not exceed pre-determined specifications[1,2]. Many methods have been described for the assay of salicylic acid in the presence of Aspirin and in Aspirin-containing analgesics[3–5], and high-performance liquid chromatography (HPLC) is of proven value for the determination of Aspirin[6,7] and for the trace determination of salicylic acid[8] and other impurities[9]. We have previously developed an HPLC method for the detection of salicylic acid in Aspirin products containing phenacetin which have had reduced turnover due to legislation restricting sales to medical prescription only[8]. Sensitivity and specificity were achieved by ultraviolet detection at 310 nm. The chromatographic separation itself, however, was only partially achieved and we now present a method which allows resolution and simultaneous quantification of Aspirin and salicylic acid in the presence of paracetamol, caffeine and phenacetin.

Substantial improvements in chromatographic performance may be also achieved by optimisation of the sample solvent composition.

In reversed-phase liquid chromatography, the sample is normally prepared as a solution in water or in the mobile phase. However, it is often more convenient to use some other miscible solvent because of stability or solubility considerations. For example, when assaying Aspirin formulations it is usually preferable to prepare solutions in methanol or a similar non-aqueous solvent to avoid hydrolysis. Similarly on occasions there may be some component of a sample matrix which is capable of altering the nature of the sample-solvent. Thus, it is important in quantitative work to consider the effect of injection technique[10] and sample solvent on peak profiles. It is well recognised that the volume of sample solvent injected is important in maintaining column efficiency and that large volumes will cause volume overload with consequent loss in performance[11]. It appears less well recognised that peak asymmetry and column efficiency are also dependent upon the *nature* of the sample solvent. With normal adsorption HPLC it has been stated that sample solvents of higher polarity than the mobile phase (used, for example, to increase solubility) yield asymmetric peaks due to band broadening at the point of injection[12]. Reversed-phase chromatography is also susceptible to these effects and it has been shown that methanol is

0021-9673/80/0000–0000/$02.25

superior to aqueous methanolic systems for aromatic hydrocarbons[13]. Small sample volumes reduce, but do not remove, these problems. When the polarities of the mobile phase and sample solvent are grossly different an extreme effect may be observed such that a single component may be eluted as two distinct peaks. Thus dihydroxybenzene isomers dissolved in methanol and eluted with water appeared as doublets[14]. Broad peaks[14] or shoulders[15] may also result. Clearly, calibration lines constructed from peak height data alone are particularly susceptible to these problems, and one possible cause for deviations from linearity[16] may be associated with sample-solvent problems.

In this paper we have illustrated the effect of sample solvent-induced peak broadening. This has important implications in quantitative analysis and it will be shown that such effects should be considered during the choice of internal standards.

EXPERIMENTAL

Apparatus

Analyses were performed using a high-performance liquid chromatograph constructed from an Altex 100A constant-flow solvent-metering pump, a Rheodyne 7120 valve injector fitted with a 20-μl loop and a Pye LC3 variable wavelength ultraviolet monitor, equipped with a 8-μl flow cell and operated at 275 nm with a sensitivity of 0.32 a.u.f.s. Chromatography was performed using a 25 cm $\times$ 4.6 mm I.D. OD2-2 column (Whatman; Partisil PXS 10/25 ODS-2) with a mobile phase consisting of acetonitrile–acetic acid–water (25:5:70, v/v/v) which was delivered at a flow-rate of 1 ml min^{-1} at a pressure of 110 bar.

Materials

Acetonitrile (HPLC grade) and methanol (analytical reagent grade) were obtained from Fisons, Loughborough, Great Britain. Acetic acid, salicylic acid, caffeine citrate, phenacetin, paracetamol, ethanol, propan-2-ol, propan-1-ol and propane-1,2-diol (all analytical reagent grade) were obtained from BDH, Poole, Great Britain. Aspirin was obtained from Reckitt and Colman, Hull, Great Britain.

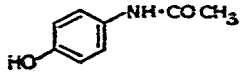
Procedures

Unless otherwise stated, the injected mixtures contained the following components in the stated concentrations: paracetamol, 0.20 mg ml^{-1}; caffeine citrate, 0.20 mg ml^{-1}; salicylic acid, 1.00 mg ml^{-1}; Aspirin, 1.00 mg ml^{-1}; phenacetin, 0.50 mg ml^{-1}. All solutions were freshly prepared and injected immediately to avoid any appreciable hydrolysis of aspirin.

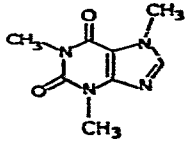
The solutions prepared in various solvents were each chromatographed at least twice, and in all cases the peak heights of the replicates were not significantly different, suggesting that the injection volumes (20 μl unless otherwise stated) were consistent. Also, since the experiments tended to take several hours to complete, care was taken to avoid changes in mobile phase composition and other instrumental parameters. As an extra precaution peak areas were determined by the cut-weigh method using traces obtained with a fast chart recorder speed (1 min cm^{-1}). The changes in peak areas were not significant throughout each experiment and so instrumental response was assumed to be constant. The coefficients of variation of

the peak areas for each experiment were measured and 2% was found to be a typical value.

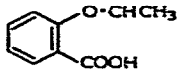
Calibration curves for each component were obtained by two methods. Initially 20-μl injections (using a fixed loop) of solutions containing various concentrations of solutes in methanol–water (25:75, v/v) were chromatographed. The experiment was repeated using a standard methanolic solution of the analgesics and in this case injections of various volumes were made using a calibrated syringe.



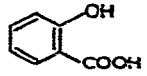
(1) Paracetamol



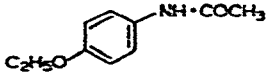
(2) Caffeine



(3) Aspirin



(4) Salicylic acid



(5) Phenacetin

RESULTS AND DISCUSSION

Typical chromatograms obtained during the analysis of the analgesic mixture are illustrated in Fig. 1. This figure shows a normal chromatogram (A) where the solutes are dissolved in mobile phase, and a comparable chromatogram (B) in a sample solvent which has considerably more acetonitrile than the mobile phase. Other sample-solvent combinations give analogous results. Thus methanol–water (30:70, v/v) gives a chromatogram similar to Fig. 1A, while methanol alone causes peak broadening and is similar to Fig. 1B. The chromatographic system described gives a good separation of all the components, and the capacity ratios were found to be: paracetamol, 1.39; caffeine, 2.35; Aspirin, 3.27; salicylic acid, 4.72; phenacetin, 6.66. It can be seen that although peak area remains constant, there is a considerable change in peak profile and peak height which may be attributed to the change in the nature of the sample solvent. The dependence of peak height upon the composition of the sample solvent is illustrated in Fig. 2 and in Table I. Little change in chromatographic performance is observed until the acetonitrile content exceeds that the mobile phase, at which point efficiency is dramatically degraded. Fig. 3 shows similar peak

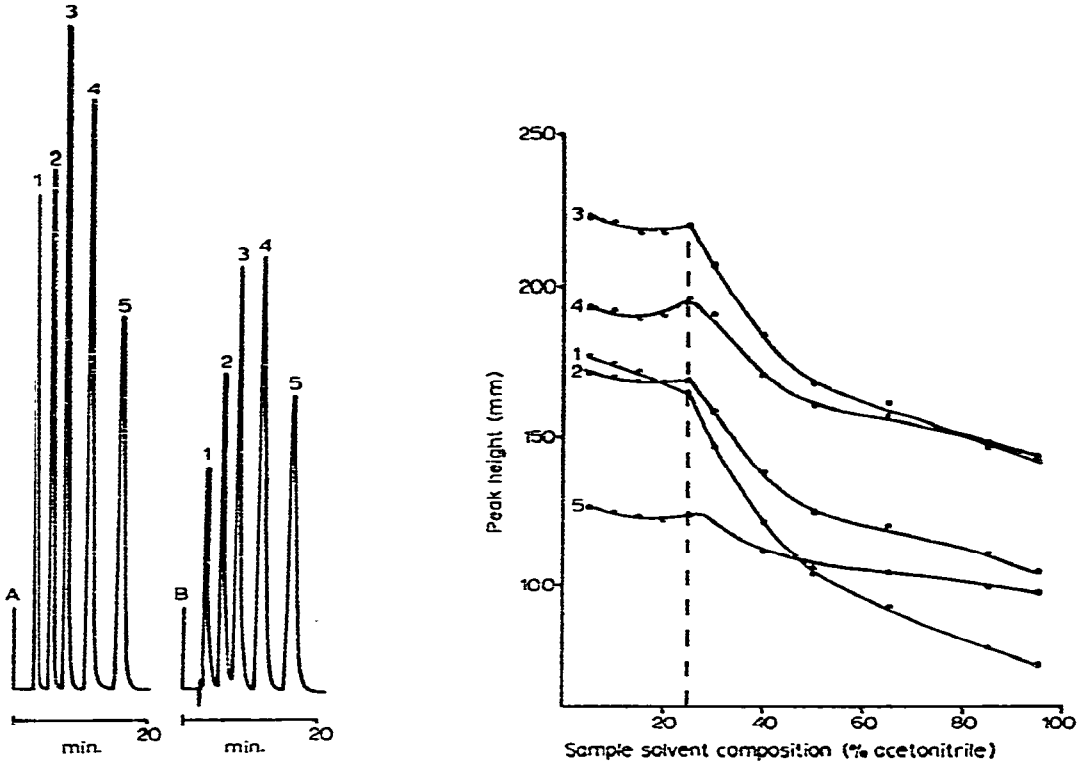


Fig. 1. Chromatograms of mixtures of paracetamol (1), caffeine (2), Aspirin (3), salicylic acid (4) and phenacetin (5). The first chromatogram (A) shows the trace obtained using mobile phase as the sample solvent (25% acetonitrile, 5% acetic acid, in water). The second trace (B) is for a mixture of identical composition prepared in an acetonitrile–acetic acid (95:5) mixture.

Fig. 2. Acetonitrile–acetic acid–water mixtures as sample solvents —the effect on peak heights. The solutes were prepared in solutions containing 5% (v/v) acetic acid and varying proportions of acetonitrile and water. The dotted line corresponds to the mobile phase composition.

TABLE I

COLUMN EFFICIENCY AS MEASURED BY THE NUMBER OF THEORETICAL PLATES FOR SAMPLE SOLVENTS OF VARIOUS COMPOSITION

Sample solvent (% acetonitrile, v/v) *	*Paracetamol*	*Caffeine*	*Aspirin*	*Salicylic acid*	*Phenacetin*
10	809	1024	1521	1418	1924
25	718	924	1400	1444	1960
40	629	855	1037	1378	1561
65	329	653	999	1220	1600
95	185	407	702	826	1363

* All the samples listed contained 5% (v/v) acetic acid and were made up to volume with distilled water.

height data for the analgesic mixture as a function of sample solvent–methanol composition. This curve exhibits a maximum at a methanol concentration of *ca.* 30% (v/v) and it is probable that at this point on the curve the polarity of the sample solvent is similar to that of the mobile phase.

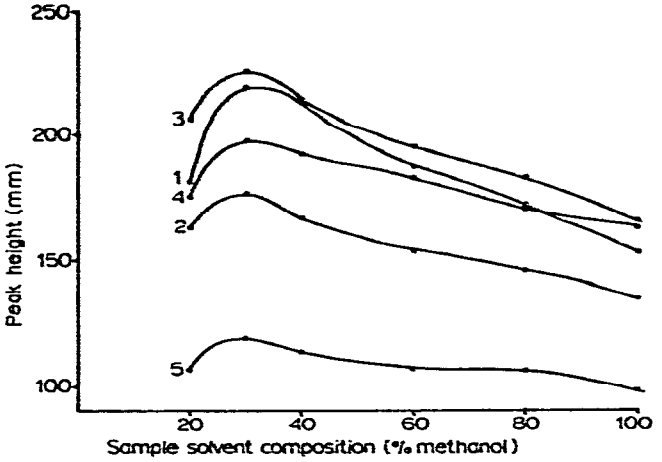


Fig. 3. Methanol–water mixtures as sample solvents —the effect on peak heights. Mixtures of the five solutes were prepared in sample solvents containing varying amounts of methanol and water. The peak height values are mean values from duplicate injections.

The causes for the observed effects are most probably associated with the length of column required for equilibration of the initial injection volume. Giddings[17] has related injection volume peak broadening to the square root of the number of theoretical plates occupied by the injected sample. It would appear that a similar mechanism is in operation here with the more polar solvents increasing the number of theoretical plates occupied by the sample and hence decreasing separation efficiency. This effect may also be observed with solvents which are less polar than the mobile phase. Fig. 4 illustrates the effect of various other sample solvents on the peak profile of salicylic acid (1 mg ml^{-1}) and shows that the peak height increases with increasing polarity[18] and that using the mobile phase as sample solvent does not necessarily maximise column efficiency.

The change in peak-height ratios which occurs, when changing sample solvent composition are important in quantitative analysis even if internal standards are used. Here it is often assumed that the peak-height ratio for two components will be dependent only upon the relative amounts of the two components. However, when the sample solvent composition changes, peak-height ratios can also change (Fig. 5). This variation may be significant and there is a potential error when the sample is of uncertain composition or where samples and standards are not prepared in the same manner. Solutes with a high capacity ratio were found to exhibit a lesser degree of peak broadening than poorly retained solutes. It is thus preferable to avoid internal standards with very short retention times and to ensure that internal standard and analyte have similar capacity ratios if sample solvent composition is liable to variation. However, provided the sample solvent is kept constant, peak height

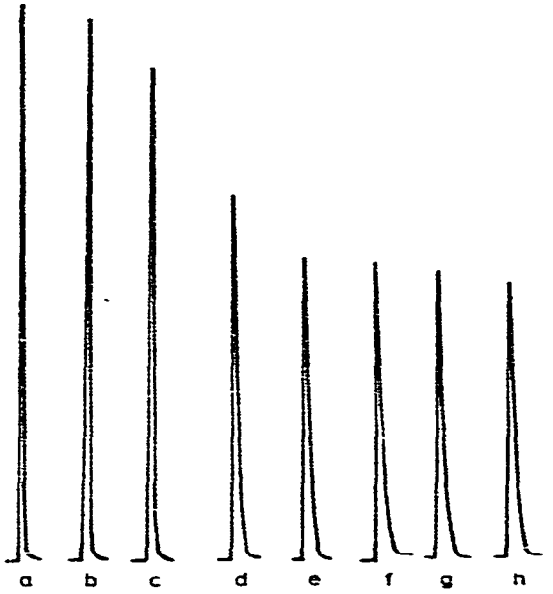


Fig. 4. The effect of various sample solvents on peak profile. Salicylic acid (1.00 mg ml^{-1}) was injected on to the column in a variety of sample-solvents. The solvents were: (a) water, (b) mobile phase, (c) propane-1,2-diol, (d) methanol, (e) acetonitrile, (f) ethanol, (g) propan-2-ol, (h) propan-1-ol.

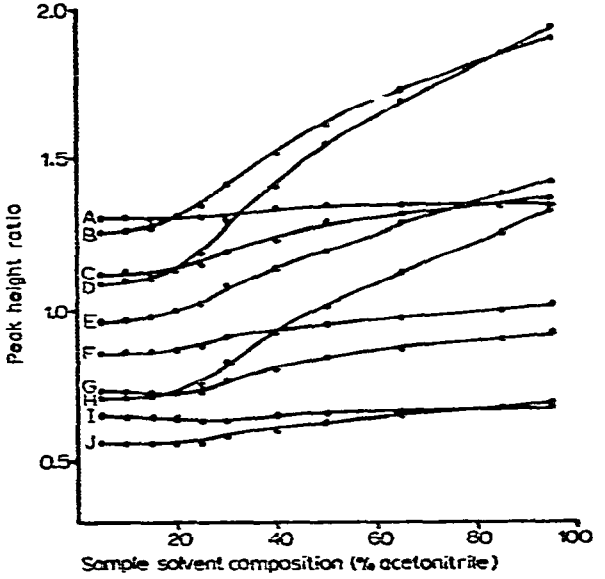


Fig. 5. The relationship between peak height ratios and sample solvent composition. A, Aspirin/caffeine; B, Aspirin/paracetamol; C, salicylic acid/caffeine; D, salicylic acid/paracetamol; E, caffeine/paracetamol; F, salicylic acid/Aspirin; G, phenacetin/caffeine; H, phenacetin/paracetamol; I, phenacetin/salicylic acid; J, phenacetin/Aspirin.

measurements produce satisfactory calibration lines. This was found to be the case when varying concentrations of the analgesics in 25% methanol were injected (constant volume, 25 μl) or when different volumes (5–20 μl) of a standard solution in methanol were analysed, although significant response differences were observed. This linearity of response indicates that small sample volume changes have an insignificant effect upon peak spreading and that little mixing of the sample solvent and mobile phase occurs. Thus provided that both standard and sample are treated in exactly the same way, quantitative measurements will be satisfactory. However, resolution and sensitivity are dependent upon the nature of the sample solvent, which may require optimisation for maximum chromatographic efficiency.

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