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Note

High-performance liquid chromatographic column switching technique in the analysis of medicated feed for an automated clean-up procedure

J. C. GFELLER* and M. STOCKMEYER

Analytical Research and Development, Pharmaceutical Department, Sandoz Ltd., CH-4002 Basle (Switzerland)

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In long-term toxicological studies, for simplicity the administration of drugs to laboratory animals is usually effected by mixing the drugs into animal feed. An analytical procedure is therefore required to determine the dosage and to ensure the homogeneity and stability of the drug substance within the feed mixture. Analysis of drug substances by high-performance liquid chromatography (HPLC) is rapid and efficient, expecially for simple feed mixtures with a high drug content[1]. However, this method is not adequate for complex mixtures as the extract must be purified before HPLC can be used. Therefore, different clean-up procedures have been proposed, *e.g.*, precipitation of chlorophyll and other components[2], liquid–liquid extraction following a clean-up step on a silica gel column[3] or simply by treatment with diatomaceous earth[4]. All procedures have their advantages but they are very time consuming and unsuitable for automation. Although the technique of column switching has been known for some time in connection with gas chromatography (GC)[5–9], this method is rather employed in HPLC[10–14] although diffusion processes are less important than in GC. The aim of this work was to demonstrate the advantages of the column switching technique in HPLC in terms of simplicity and automation possibilities with commercially available instrumentation for the routine analysis of pharmaceuticals in complex animal feed mixtures.

EXPERIMENTAL

Apparatus

The chromatographic system consists of two Altex Model 110 pumps (Altex Scientific, Berkeley, CA, U.S.A.), an automatic sample injector, home-made and subsequently adopted commercially (ASI 45, Kontron, Zurich, Switzerland), to which an additional six-port valve (7010-A, Rheodyne, Berkeley, CA, U.S.A.) was fitted in series. A LiChrosorb RP-8 column with 5-μm particles (25 × 0.46 cm I.D., Knauer, Oberursel, G.F.R.) for the analytical work and a LiChrosorb RP-18 column with 10-μm particles (5 × 0.46 cm I.D., Knauer) for the sample clean-up are used. The injection and switching valve are controlled with two timers of the sample injection unit, as shown in Fig. 1, which is also suitable for an automated pre-column derivatization technique mentioned earlier[15]. For detection a Model LC55 UV monitor

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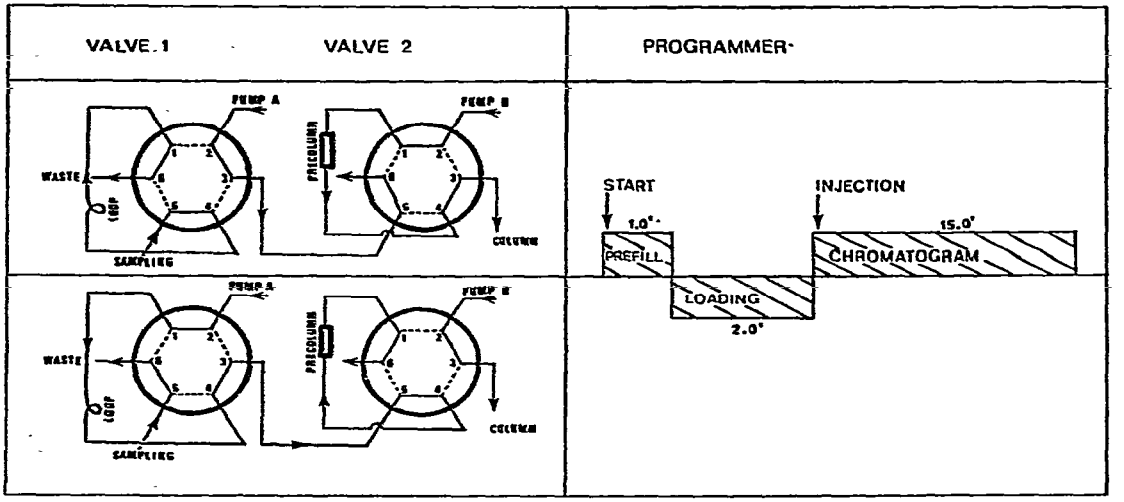

Fig. 1. Schematic diagram of the column switching assembly. Valve 1 + programmer, automated sample injector; valve 2, column switching valve; pre-column, LiChrosorb RP-18, 10 μm (5 × 0.46 cm); analytical column, LiChrosorb RP-8, 5 μm (25 × 0.46 cm). Mobile phases: pump A, water, 0.2 ml/min flow-rate (clean-up mobile phase); pump B, acetonitrile–water (55:45), 1.5 ml/min flow-rate.

(Perkin-Elmer, Norwalk, CT, U.S.A.) at a wavelength of 240 nm (UV maximum of the drug substance) was used; the injection volume was 20 μl in all instances.

Reagents

The animal feed consisted of a complex mixture of cereals, plant and animal proteins, mineral substances, amino acids and vitamins (feed 850 G4, Nafag, Gossau, Switzerland). The drug compound to be tested toxicologically was Fluorproquazone (Fig. 2), which has a UV maximum at 240 nm. For extraction of the medicated feed methanol ("zur Synthese", Merck, Darmstadt, G.F.R.) proved to be optimal. For the mobile phases, methanol and acetonitrile (HPLC grade) (Rathburn Chemicals, Walkerburn, Great Britain) were used as indicated in the text or figures.

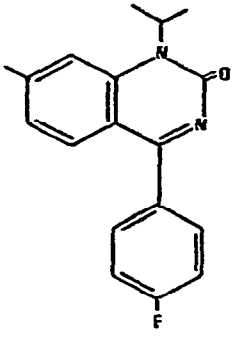

Fig. 2. Structure of Fluorproquazone.

Extraction procedure

Fifty grams of medicated feed were transferred into a 150-ml glass-stoppered flask, 100 ml of methanol were added and the mixture was stirred with a magnetic stirrer for 30 min. The brown suspension was decanted, centrifuged for 15 min (1800 *g*) and an aliquot of the clear supernatant was used for HPLC injection.

RESULTS AND DISCUSSION

Chromatographic procedure

Injection of the methanolic solution on to the analytical column, *i.e.*, without a clean-up step, is shown in Fig. 3. As can be seen, not only Fluorproquazone cannot be resolved, but the blank itself gives a different peak pattern after each injection. The reason for this behaviour is the large amount of extracted compounds resulting in overloading of the column, hence influencing the chromatographic properties of the stationary phase. Also, the injection of a pure methanolic sample solution provides an

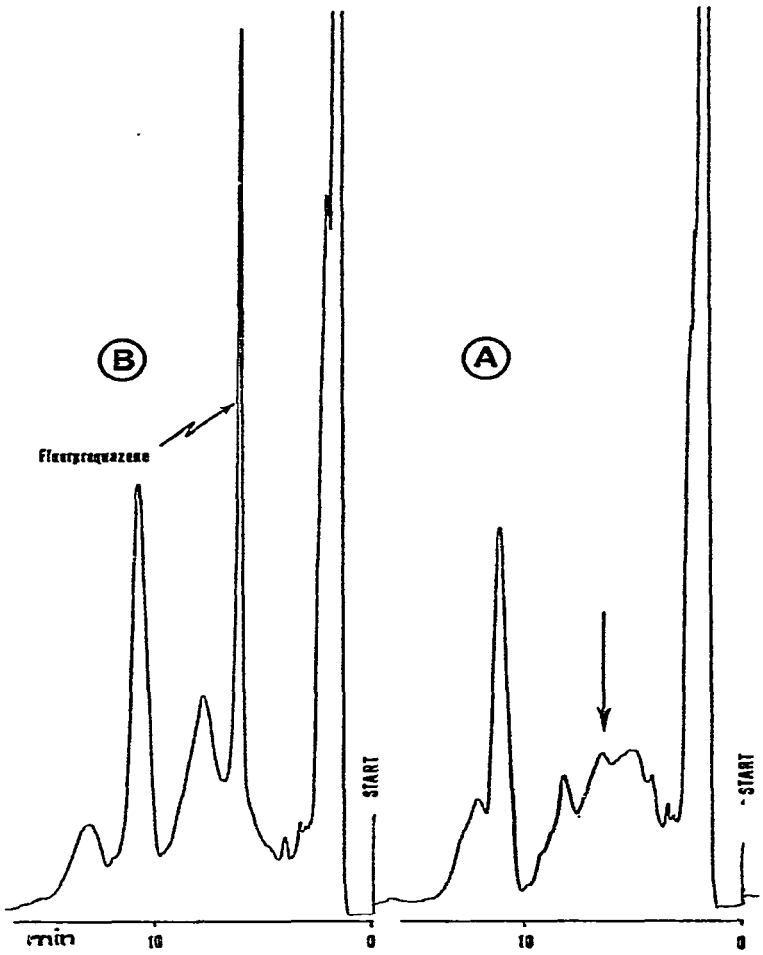

Fig. 3. Chromatogram of a medicated feed sample without clean-up procedure (mobile phase of analytical column as in Fig. 1). A, Blank solution; B, sample solution.

increased elution force on the top of the analytical column. Therefore, samples should be diluted, *e.g.*, with water, prior to injection or an extraction medium with higher polarity should be used, but this can cause problems with respect to complete extraction.

For these reasons, a column switching technique appeared to be advantageous, particularly in view of an automated sample treatment as proposed in Fig. 1. A back-flush procedure according to Huber and Becker[10] was chosen because of the simple handling involved and its ability to separate interfering polar substances from the drug substance to be analysed[14].

To demonstrate the performance of the clean-up step, injected sample extracts were treated on the pre-column with different methanolic mobile phases before separation on the analytical column. As can be seen from Fig. 4, 80% methanol–water as

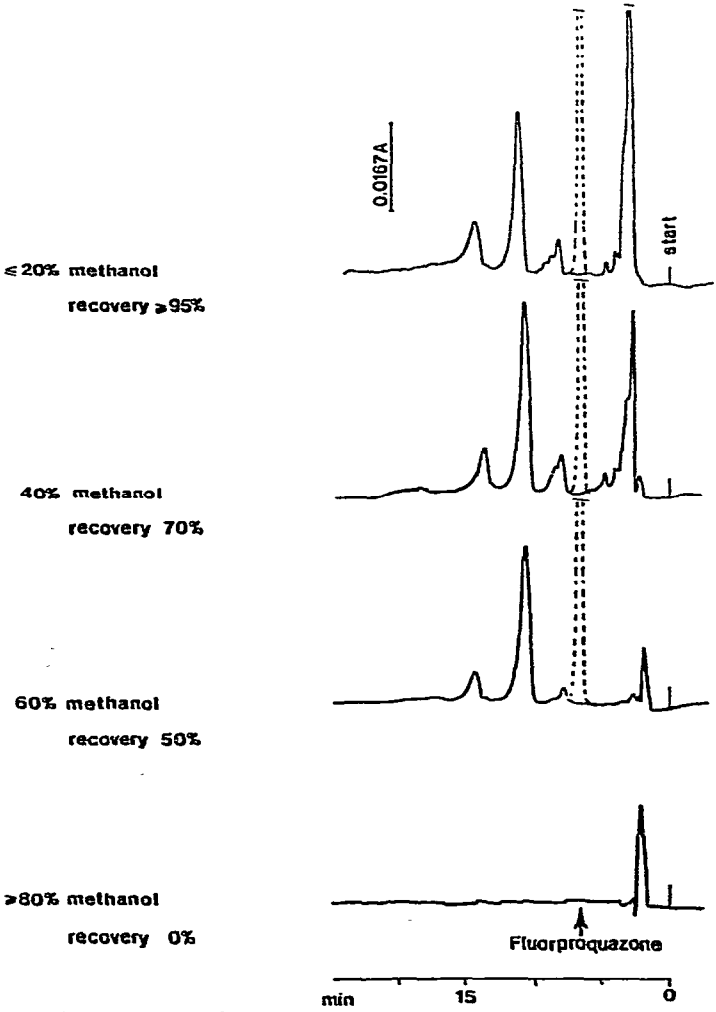

Fig. 4. Influence of composition of the clean-up mobile phase (pump A) on the recovery of fluorproquazone and the blank.

the mobile phase eliminates almost all of the interfering substances, but unfortunately Fluorproquazone is also eluted from the pre-column. We found that the moderately polar Fluorproquazone is retained quantitatively only with a mobile phase containing less then 40% of methanol. Because the blank does not increase with decreasing methanol content, pure water was used for the clean-up procedure (Fig. 5).

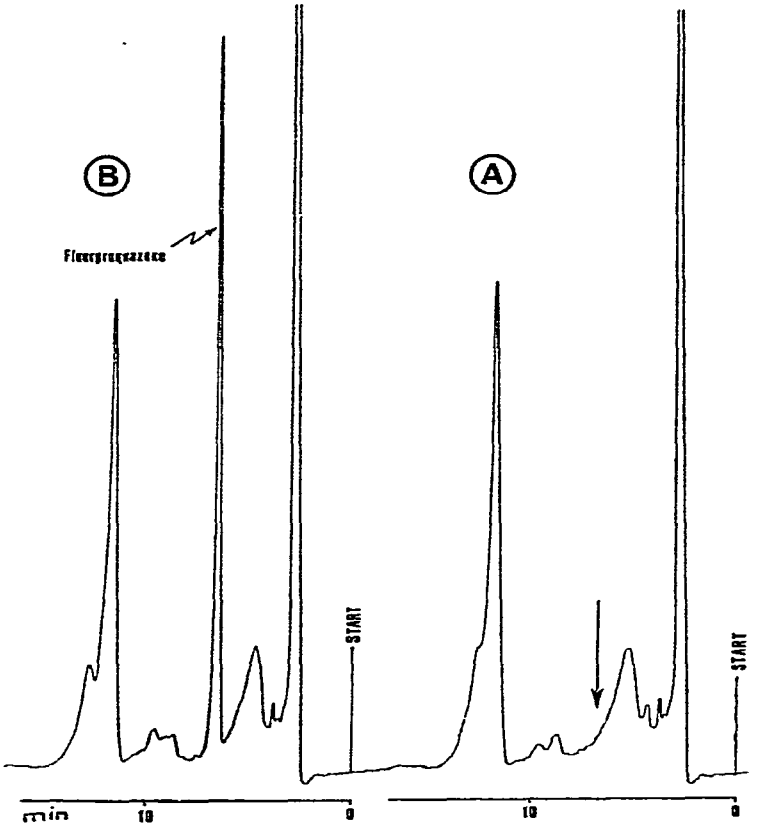

Fig. 5. Chromatogram of a medicated feed sample (118 ppm of Fluorproquazone with a column switching clean-up procedure. Mobile phases as in Fig. 1. A, Blank solution; B, sample solution.

The unavoidable peak broadening arising from the column switching was observed with a standard solution and was measured as *ca.* 11 sec (ca. 25%), still ensuring a good resolution. Sample solutions could not be compared because the Fluorproquazone peak is broadened by column overloading derived from the blank. The elution time from the pre-column can be set by the automated injector (Fig. 1), and was found to be optimal at 2 min with respect to time and clean-up. Other stationary phases (RP-8, DIOL) were studied, but reversed phase RP-18 was found to be the most efficient.

Validation of the assay of Fluorproquazone in animal feed

Calibration line and limit of detection. To 50-g feed samples 0, 1, 3, 5, 6, 8 and 10 mg of Fluorproquazone (0–200 ppm) dissolved in an aliquot of methanol were

added, shaken for 5 min and, after standing for 1 h, extracted according to the previously mentioned procedure. The clear sample solutions were injected and a linear concentration graph could be constructed from the peak height ($r = 0.9999$). A positive intercept indicated a blank peak in the range of about 1 ppm of Fluorproquazone. The limit of detection can be estimated to be about 5 ppm and is, of course, influenced by the different lots of feed used. This result is highly satisfactory because the lowest concentration to be analysed is about 100 ppm.

Precision of the assay. Amounts of 59 and 118 ppm of Fluorproquazone were added to feed samples and five and six samples, respectively at each concentration were taken through the extraction and HPLC procedure. Peak height measurements showed a relative standard deviation of 0.7% at 59 ppm and 0.8% at 118 ppm.

Recovery of Fluorproquazone from feed. Standard samples and "spiked" feed extracts containing 118 ppm of Fluorproquazone were prepared as described above and their peak heights were compared. Values for five replicate experiments were calculated using the equation

$$R(\%) = \frac{\text{Peak height (extract)}}{\text{Peak height (standard)}} \cdot 100$$

and a recovery $R \geqslant 95\%$ was found in each instance.

Stability of Fluorproquazone in feed. A sample was stored at −25°C and compared with a sample stored in the animal cage at +23°C for 2 weeks. The concentration was calculated with freshly prepared calibration samples. Fluorproquazone remained both stable and extractable during the tested period, giving mean values ($n = 3$) of 97.3% (−25°C) and 96.8% (+23°C).

Application of the method. The technique was applied to the content uniformity test with Fluorproquazone in feed in a batch of 10 kg at a 118 ppm concentration. Six samples were assayed as described and the results indicate that the mixture is perfectly homogeneous (Table I).

TABLE I

CONTENT UNIFORMITY OF A MEDICATED FEED BATCH (118 ppm OF FLUORPROQUAZONE)

Sample No.	*Fluorproquazone with respect to declared content, % ($n = 2$)*
1	97.7
2	97.7
3	98.3
4	93.3
5	93.3
6	95.2
Average: $\bar{x} = 95.9$	

CONCLUSIONS

The column switching technique for sample clean-up treatment is very efficient

for the determination of Fluorproquazone in medicated feed. The method is simple, sensitive, reproducible and very rapid, especially through automation with commercially available equipment. For these reasons we have introduced this method routinely in our laboratories for the analysis of other drug substances in medicated feed.

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