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# OPTIMISATION OF A REVERSED-PHASE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC METHOD FOR THE DETERMINATION OF FLAVONE ACETIC ACID AND ITS MAJOR HUMAN METABOLITES IN PLASMA AND URINE

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## SUMMARY

A high-performance liquid chromatographic method for the determination of flavone acetic acid (FAA) and its major human metabolites in plasma and urine is described. Two factors were identified as being the key to resolving the metabolites; pH and buffer ionic strength. Run at optimal conditions of 10 m*M* ammonium acetate, pH 5.5–propan-2-ol (80:20) and a column temperature of 40°C on a μBondapak $C_{18}$ 10 μm particle column (30 cm × 3.8 mm I.D.), two major metabolites were identified [FAA, retention time ($t_R$) 6.02 min ± 0.5% coefficient of variation (C.V.); metabolite 1, $t_R$ 4.13 min ± 1.1% C.V.; metabolite 2, $t_R$ 5.10 min ± 0.5% C.V. and hesperidin, internal standard, $t_R$ 4.69 min ± 1.6% C.V.]. A solid-phase technique using Bond Elut $C_2$ 40-μm particles is described which extracts FAA, metabolites and internal standard with efficiences in excess of 90%. Considerable attention has to be paid to sample preparation: FAA has poor aqueous solubility at acidic pH and the metabolites degrade back to FAA via intermediates at alkaline pH. Both problems can be avoided by buffering and diluting samples with 10 m*M* ammonium acetate, pH 5.5.

## INTRODUCTION

Flavone acetic acid (FAA, Fig. 1) is a parent member of a potentially new class of anticancer drugs, based on the naturally occurring flavone ring structure, cur-

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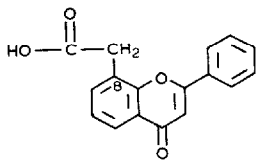

Fig. 1. Molecular structure of flavone acetic acid (FAA).

rently undergoing extensive clinical trials in both Europe and the United States. Preclinical animal trials showed that the drug possessed unusual pharmacologic properties: it produces none of the conventional toxicities associated with cytotoxic chemotherapy; it is active only in slowly growing solid tumours; it can stimulate natural killer cell activity and synergise with interleukin II; and a therapeutic window of plasma concentration has to be achieved before activity is seen [1–3]. Results from clinical trials to-date have shown (i) a complete lack of activity in human cancer, (ii) different toxicity profiles at different dose schedules and (iii) non-linear pharmacokinetics [4–6].

In an attempt to understand and explain some of these properties and the species differences, we are investigating the metabolism of this drug in both man and mouse. In this report we describe the optimisation of a rapid high-performance liquid chromatographic (HPLC) assay which resolves the major human metabolites of FAA into two peaks and a solid-phase sample preparation technique which extracts FAA and metabolites along with a choice of two internal standards from plasma and urine with high efficiency.

## EXPERIMENTAL

### *Apparatus*

The liquid chromatograph used was a Hewlett-Packard Model 1090 equipped with: a PV5 ternary, low-pressure mixing, solvent delivery system; a variable-volume 1–25 μl automatic injector (set at 20 μl) and autosampler; a heated column compartment and a multi-diode rapid scanning UV–visible spectrophotometric detector (Hewlett-Packard Analytical, Manchester, U.K.). System control, data collection and data evaluation were performed by a Hewlett-Packard Series 9000 300 ("Chemstation") computer with 40 megabytes of hard Winchester Disk memory, a "think jet" printer and "color-pro" plotter, all linked through HP-IB interfaces to the liquid chromatograph. The chemstation operating software written in Pascal includes an integration package.

### *Chemicals, reagents and drug standard solutions*

All solvents used in liquid–liquid extraction experiments were analytical reagent grade (AnalaR, BDH, Poole, U.K.). All methanol and propan-2-ol were HPLC grade (Rathburn Chemicals, Walkerburn, U.K.). Ammonium acetate, acetic acid and ion-pairing agents were HPLC reagent grade (Fisons, Loughborough, U.K.). Water was deionised and bidistilled in a quartz glass still and all other reagents, chemicals and solvents were of the highest grade available com-

mercially. Pure FAA (free acid) was from LIPHA Lyonnaise Industrielle (Lyon, France); hesperidin (HESP, internal standard) was a kind gift from Aldrich (Gillingham, U.K.) and hesperitin (internal standard) was from Sigma (Poole, U.K.). Standards of FAA metabolite 1 (M1) and metabolite 2 (M2) were obtained in our own laboratory after purification from patient urine specimens. Whilst full details of the isolation, purification and characterisation of the two metabolites will appear in another report, our preliminary evidence suggests that both are glucuronide conjugates.

Standard solutions of FAA were prepared by dissolving a known weight in a small volume of dimethylsulphoxide (DMSO, Sigma) and further diluting with 10 m*M* ammonium acetate, pH 5.5, to give a concentration range of 500 $\mu$g/ml to 50 ng/ml. Likewise, HESP was solubilised in DMSO and diluted in 10 m*M* ammonium acetate, pH 5.5. Metabolites in the concentration range of 250 $\mu$g/ml to 50 ng/ml were made up in 10 m*M* ammonium acetate, pH 5.5. All standard solutions were stored in glass autosampler vials with aluminium caps and PTFE liners at 4°C and were periodically made up fresh. Calibration curves were constructed by injecting 20 $\mu$l of standard to yield a range of 1 ng to 10 $\mu$g on the column, and these were stored by the computer for quantitation of unknowns by an external standard method. Limit of detection was set at the 3:1 peak height-to-baseline noise ratio.

*Chromatographic conditions*

The stationary phase was $\mu$Bondapak $C_{18}$ 10-$\mu$m irregular particles obtained prepacked in 30 cm × 3.8 mm I.D. stainless-steel columns from Waters Assoc. (Northwich, U.K.). The mobile phase consisted of 10 m*M* ammonium acetate adjusted to pH 5.5 with acetic acid–propan-2-ol (80:20). Elution was isocratic at a flow-rate of 1 ml/min. Mobile phase composition was maintained accurately at the above proportions by low-pressure solvent mixing of the two components and kept permanently degassed by continuous sparging with helium during chromatography. Mobile phase components were also filtered prior to helium degassing. The column was maintained at a constant temperature of 40°C. Finally, chromatographic peaks were monitored at 303 nm, the UV maximum wavelength for FAA and its metabolites (see Fig. 5).

*Extraction procedure*

Blood (immediately separated into plasma) and urine specimens were from patients who either received 4.8 g/m$^2$ FAA as a 1-h infusion or 8.6 g/m$^2$ FAA as a 6-h infusion during a phase I clinical trial and were immediately frozen or buffered and then frozen (see Results, *Patient sample preparation and analysis*). FAA and its metabolites were extracted using 500 mg of Bond Elut $C_2$ 40-$\mu$m irregular particles packed into 2.4-ml reservoirs placed in a Vac Elut (ten place) vacuum manifold attached to a vacuum pump (Analytichem International, supplied by Crawford Scientific, Strathaven, U.K.).

Freshly thawed urine or plasma (1–2 ml) was diluted and buffered to pH 5.5 with 10 m*M* ammonium acetate (see Results, *Patient sample preparation and analysis*). HESP (1–100 $\mu$g) was added as an internal standard. The mini-col-

umns were solvated under negative pressure with 2 ml of methanol, then washed with 2 ml of water. Immediately, plasma or urines (1–2 ml) were loaded slowly on to the columns, which were then washed with 2 × 2 ml water, and finally FAA and metabolites were eluted in 1 ml methanol. A 100-$\mu$l volume of 4 *M* ammonium acetate, pH 5.5, was added to the methanol and samples were centrifuged at 1000 *g* for 5 min. Thereafter, supernatants were ready for either direct injection or further concentration if required.

## RESULTS AND DISCUSSION

### *High-performance liquid chromatography*

The aim of this present study was to develop a rapid HPLC assay which would be capable of identifying all of the, as yet unidentified, human metabolites of FAA. To achieve this aim we adopted two approaches: (i) using direct injections of diluted patient urines as a source of metabolites we attempted to resolve chromatographically all the metabolites into single component peaks and (ii) we attempted to purify the metabolites and then use them as standards to develop a separation.

In our initial studies with direct urine injections only one metabolite peak was detected. Two factors altered the situation dramatically: addition of 10 m*M* ammonium acetate as an aqueous modifier and pH. The extent of the influence of pH on the retention times of FAA and its metabolites is illustrated in Fig. 2. In the pH range 4.5–6.0, the metabolites resolved into two peaks. At acidic pH, the pH at which most published assays operate, the parent drug eluted late on and the metabolites eluted unresolved, and this is exactly what others have noticed [6,7]. Above pH 6.0 the metabolites co-eluted with the parent drug. Optimum conditions for the rapid separation of parent drug and metabolites were established to be pH 5–5.5 with 10 m*M* ammonium acetate–propan-2-ol (80:20) at a temperature of 40°C. Chromatographic characteristics of this separation are con-

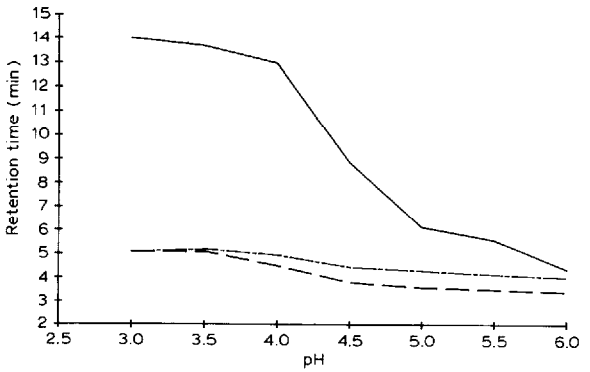


Fig. 2. Influence of pH on the retention times of FAA and its metabolites. (———) FAA; (- - -) metabolite 1; (- - - - -) metabolite 2. Chromatographic conditions are as described in Experimental except that the 10 m*M* ammonium acetate component of the mobile phase was adjusted to the desired pH (3–6 in 0.5 unit steps) with acetic acid.

TABLE I

HPLC OF FAA AND ITS METABOLITES

Chromatographic conditions as described in Experimental.

| Compound | Retention time, $t_R$ (min) ± C.V. (%) | Capacity factor, $k'$ | Efficiency, $N$ ($m^{-1}$) | Resolution ($R_s$) | | | Quantitation linearity* (1 ng to 10 μg) | |
|---|---|---|---|---|---|---|---|---|
| | | | | Compared with FAA | Compared with M2 | Compared with HESP | $y=mx+c$* | $r^2$ |
| M1 | 4.13 ± 1.1 | 0.87 | 11 400 | 4.8 | 1.8 | 1.6 | $y=40x+5.7$ | 0.997 |
| HESP | 4.69 ± 1.6 | 1.13 | 11 000 | 3.2 | 1.0 | – | | |
| M2 | 5.10 ± 0.5 | 1.32 | 5 800 | 1.6 | – | | $y=45x-2.5$ | 0.995 |
| FAA | 6.02 ± 0.5 | 1.73 | 13 500 | – | | | $y=48x-3.8$ | 0.999 |

*$y$ = peak area; $x$ = amount injected; $c$ = intercept with $y$-axis; $m$ = integrator response factor.

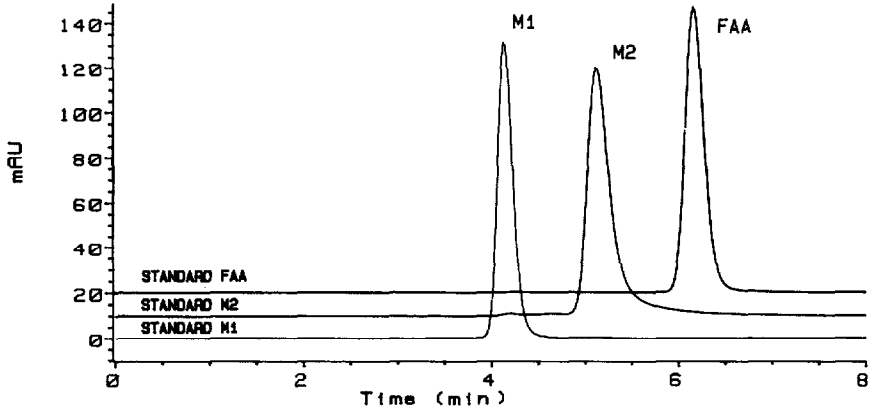


Fig. 3. Chromatograms of standard solutions of 50 μg/ml FAA and metabolites. Chromatographic conditions as in Experimental and peaks were monitored at 303 nm.

tained in Table 1. The lower efficiency of metabolite 2 is due to poor peak symmetry suggesting that it may possibly still contain more than one component. In Fig. 3 is a chromatogram of M1, M2 and FAA standards run using the method described. The separation was extremely stable with retention times ($t_R$) varying by only 1% during the day and reproducible from day to day. Limit of detection was 1 ng on-column and calibration curves over a wide range of standards (1 ng to 10 μg) were linear with $r^2$ close to 1.0 (Table I). Calibration curves were almost identical for FAA and metabolites and in the absence of standards a calibration curve for FAA could be applied with some confidence to the accurate determination of the metabolites.

*Extraction technique*

Two candidates, both naturally occurring flavonoids and therefore structural analogues of FAA, were investigated as possible internal standards, and both were found to be suitable. Hesperitin eluted late on with a retention time of 13.4 min and its inclusion greatly lengthens analysis time. However, certain applications may require its use. For instance, in mice a metabolite of FAA elutes close to our

other preferred internal standard for the analysis of patient samples, HESP (see Table I, $t_R$ mouse metabolite 4.35 min, $t_R$ HESP 4.69 min). HESP is a gluconated more polar analogue of hesperitin which occurs naturally in orange peel; its chromatographic properties and extraction behaviour made it an ideal internal standard. In Fig. 4 is a chromatogram of a plasma extract spiked with 100 $\mu$g of hesperidin and FAA and in Fig. 5 is the UV absorption spectrum of FAA and HESP. FAA has maxima at 256 and 303 nm. The latter was chosen as the optimum wavelength permitting both high sensitivity (down to 1 ng on-column) and high selectivity (see Figs. 4 and 6). Although HESP has a $\lambda_{max}$ of 284 nm, it still absorbs significantly at the monitor wavelength of 303 nm. Peak height/area ratios of equal amounts of FAA to HESP were approximately 4:1. The solid-phase extraction technique yielded clean samples with very little interference from plasma and virtually none from urine even at high sensitivity (Fig. 6). Efficiencies were high, in excess of 90% for all components including the internal standard (Table II), and linear over a wide range of concentrations encompassing those normally expected in clinical specimens (Table II).

A survey of extraction techniques currently employed in FAA analysis, both clinical and preclinical, reveals that the majority are based on a variety of liquid–liquid procedures: extraction in diethyl ether after acidification with 6 *M* hydrochloric acid [4], extraction into chloroform–propan-2-ol at pH 2 [7] and extraction into chloroform after acidification with 5% trichloroacetic acid [6]. In a series of preliminary experiments we studied the influence of several factors on the extraction of FAA and its metabolites. Briefly, the metabolites showed little solvent specificity. Positively charged ion-pairing agents improved the situation but could still only achieve a maximum of 40% efficiency, which was similar to the best results obtained with acidification. Thus, we feel that liquid–liquid techniques are inappropriate for the metabolites and recommend a solid-phase method where extraction efficiencies in excess of 90% can be routinely achieved.

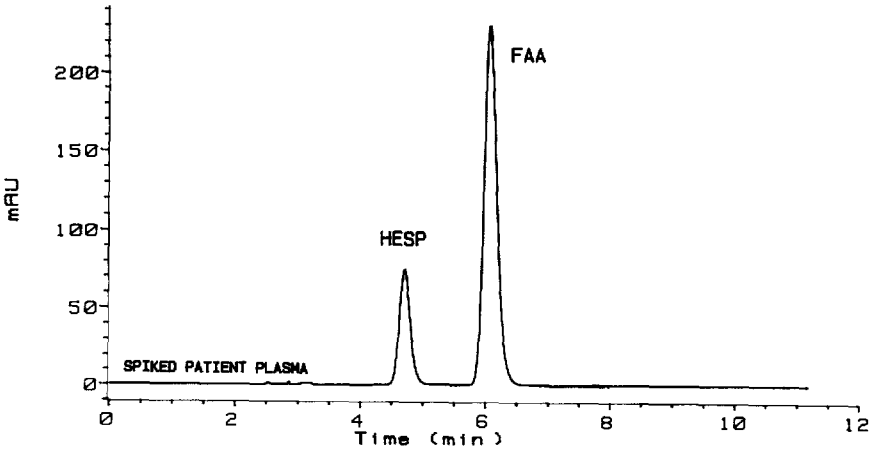


Fig. 4. Chromatogram of an extracted patient plasma spiked with 100 $\mu$g/ml FAA and HESP (internal standard). Chromatographic conditions and solid-phase extraction procedure as in Experimental.

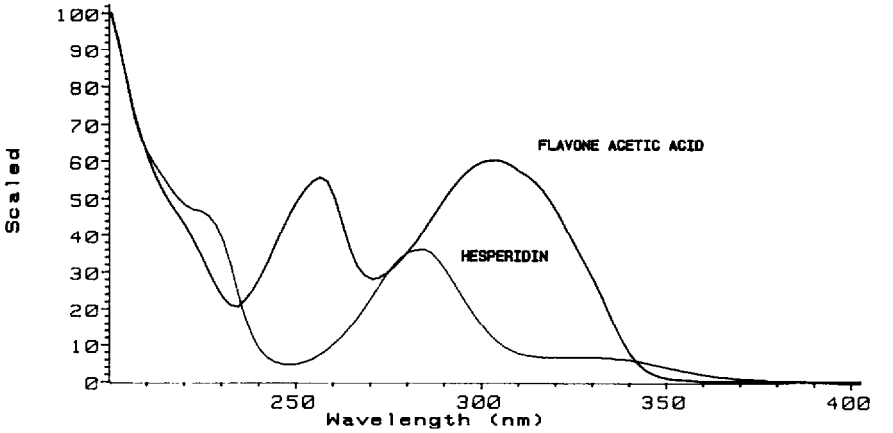


Fig. 5. UV absorption spectrum of HESP and FAA. Both spectra have been normalised, scaled and superimposed and are, therefore, directly comparable. At the monitor wavelength of 303 nm, FAA has approximately four-fold greater UV absorption than HESP.

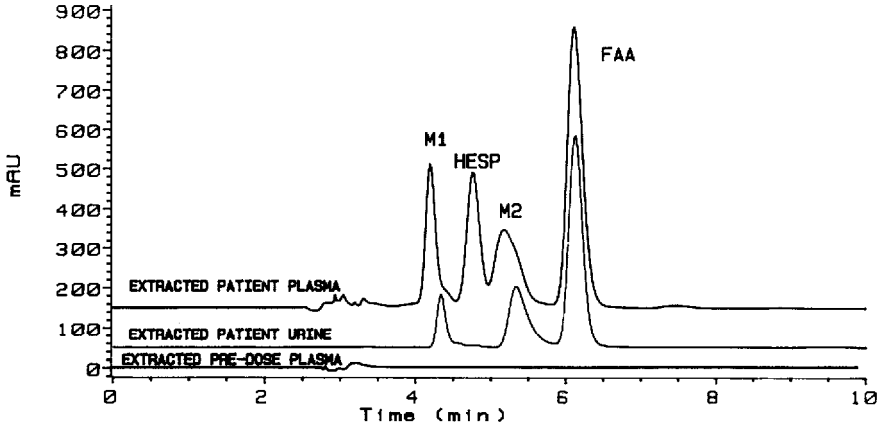


Fig. 6. Chromatograms of patient samples extracted and run using the procedures described in Experimental. Upper trace: plasma from patient taken 12 h after 8.6 g/m$^2$ FAA as a 6-h infusion: FAA 140 μg/ml, M1 65 μg/ml and M2 50 μg/ml. Middle trace: urine collected over the first 6 h after 4.8 g/m$^2$ as a 1-h infusion: FAA 5.0 mg/ml, M1 0.9 mg/ml and M2 2.3 mg/ml. Lower trace: extracted predose plasma.

*Patient sample preparation and analysis*

Two protocols have been recommended for phase II clinical trials of FAA: 4.8 g/m$^2$ as a 1-h infusion and 8.6 g/m$^2$ as a 6-h infusion. At these extremely high doses plasma concentrations of FAA can approach 1 mg/ml after the 1-h infusion and 0.5 mg/ml after the 6-h infusion [6] and urine concentrations can range up to ten times greater. Fig. 6 shows a chromatogram of a plasma sample from a patient who received the 6-h infusion and a urine sample (without internal standard) from a patient who received the 1-h infusion.

Two factors have to be taken into account prior to sample storing and processing: the low stability of the two metabolites at alkaline pH and the poor solubility of the parent drug at acidic pH. The pH instability of M1 and M2 is illustrated

TABLE II

SOLID-PHASE EXTRACTION OF FAA AND ITS METABOLITES FROM PLASMA USING 500 mg $C_2$ 40-$\mu$m SILICA PARTICLES (BOND ELUT) PACKED IN 2.4 ml CAPACITY MINI-COLUMNS

Blood bank plasma samples (1 ml) were spiked with varying amounts of FAA and metabolites and extracted using the procedure described in Experimental.

| Compound | Reproducibility (100 $\mu$g) ($n$=20), mean (%) ± C.V. (%) | Linearity* (500 $\mu$g to 100 ng) ($n$=10) | |
|---|---|---|---|
| | | $y = mx + c$ | $r^2$ |
| M1 | 90.3 ± 14.4 | $y = 0.84x + 0.9$ | 0.995 |
| HESP | 95.2 ± 4.8 | | |
| M2 | 90.9 ± 7.6 | $y = 0.93x - 0.80$ | 0.999 |
| FAA | 93.8 ± 9.4 | $y = 0.90x + 0.94$ | 0.999 |

*$m$ = extraction efficiency; $c$ = intercept with $y$-axis; $r^2$ = regression correlation coefficient.

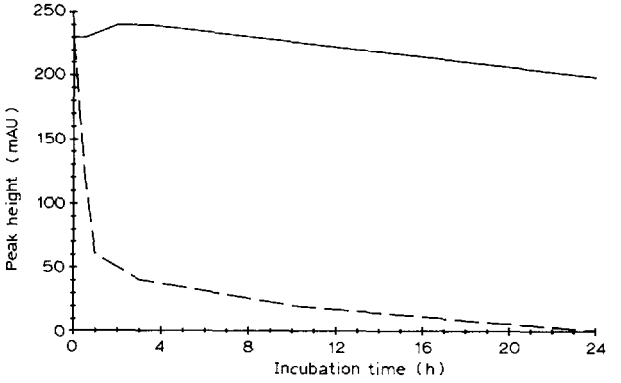


Fig. 7. Stability of 100 $\mu$g/ml M1 incubated at 37°C and pH 5.5 (———) or pH 9.0 (- - -). Degradation was assessed by measuring the reduction in peak height with time. Half-life at pH 9.0 was 30 min.

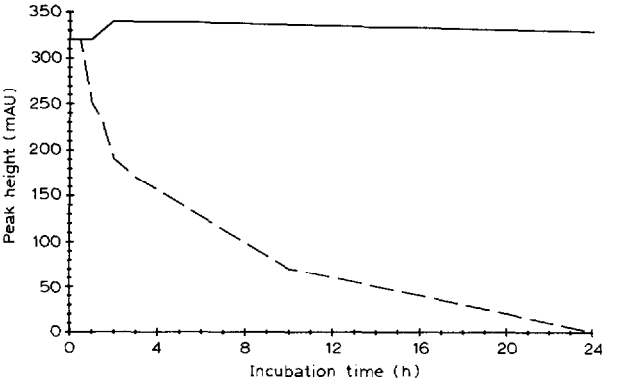


Fig. 8. Stability of 200 $\mu$g/ml M2 incubated at 37°C and pH 5.5 (———) or pH 9.0 (- - -). Degradation was assessed by measuring the reduction in peak height with time. Half-life at pH 9.0 was 4 h.

in Figs. 7 and 8 and it should be noted that even at pH 5.5 M1 starts to degrade at 37°C. At pH 9.0 and 37°C the half-life of M1 and M2 is 30 min and 4 h, respectively. A most significant observation is that M1 and M2 degrade back to the parent drug through intermediate stages. Therefore pH stability is important to avoid metabolite artefacts being introduced into samples or an overestimation of the parent compound occurring. This is particularly important for urine specimens where in clinical trials in order to prevent any possible crystallisation of the parent drug within the kidney tubules sodium bicarbonate is infused to induce alkaline diuresis and urinary pH values in excess of 8.0 have been recorded.

We have estimated the maximum solubility of FAA at pH 4.0 in aqueous solutions to be 100 $\mu$g/ml. Left unattended, even at pH 8.0, significant precipitation of FAA from urine occurs within one or two days stored at 4°C.

To overcome solubility and stability problems, we propose a number of precautions. Plasma samples should be immediately frozen and only after thawing and prior to extraction should they be buffered and diluted. For the earlier time points in pharmacokinetic trials we suggest a 1:10 dilution with 10 m*M* ammonium acetate, pH 5.5, and at later time points a 1:2 dilution. Urine specimens should be buffered and diluted before storage by a factor of 1:50 with 10 m*M* ammonium acetate, pH 5.5, as soon as they are collected.

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