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The analysis of SK&F 94120, a novel inotropic agent, and its four metabolites by isolation on C18 AASP cassettes followed by high-performance liquid chromatography

J. C. PEARCE, W. J. LEAVENS, J. A. JELLY, K. A. FERNANDES and R. D. McDOWALL*

Department of Drug Analysis, Smith Kline and French Research Ltd., The Frythe, Welwyn, Herts AL6 9AR, UK

Abstract: A selective and specific assay for SK&F 94120 [5-(4-acetamidophenyl)pyrazin-2(1H)-one] and its four metabolites in plasma has been developed. The method incorporates a single liquid–solid extraction step using C18 Analytichem Automated Sample Processor (AASP) cassettes. This is followed by successive elutions of the solid phase with two mobile phases of increasing acetonitrile content, by an AASP liquid chromatography module.

A mobile phase containing 10% acetonitrile elutes a glucuronide metabolite from the cartridge which is then chromatographed and quantified intact. A second mobile phase, containing 20% acetonitrile, is then used to elute the unchanged drug and the three other metabolites from the same cartridge.

The assay shows good accuracy and precision (less than 10% for all analytes) and is able to determine SK&F 94120 and its metabolites in plasma at concentrations between 0.05 and 1.00 mg l^{-1}.

Keywords: *Reversed-phase chromatography; liquid–solid extraction; AASP; glucuronide SK&F 94120 and metabolites.*

Introduction

SK&F 94120 [5-(4-acetamidophenyl)pyrazin-2(1H)-one], is a novel, potent, orally active inotropic agent with vasodilator activity considered useful in the treatment of congestive heart failure [1]. The metabolism of the compound in the rat and dog is shown in Fig. 1. The major metabolite in the rat and dog is an *O*-glucuronide conjugate (SK&F 94120-MET I) which circulates in the plasma and is excreted from the body via urine and faeces. The parent drug is also metabolised by gut microflora, during passage through the gastro-intestinal tract, to 2-(4-acetamidophenyl)pyrazine (SK&F 94120-MET II); this compound is absorbed and further metabolised by the hepatic microsomal oxidase system to either 3-(4-acetamidophenyl)pyrazine-1-oxide (SK&F 94120-MET III) or 2-(4-aminophenyl)pyrazine (SK&F 94120-MET IV) [2].

* Author to whom correspondence should be addressed.

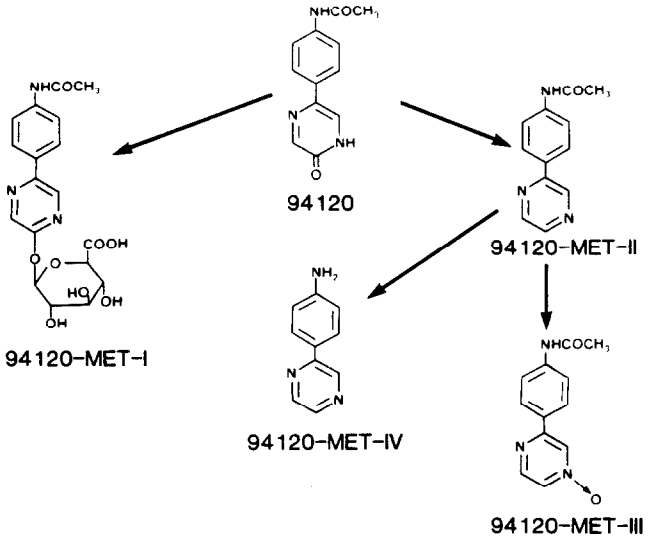

Figure 1
SK&F 94120 metabolic pathway.

During the development of any drug, a sensitive and specific assay is required in order to monitor the time course of absorption and elimination of the compound, to define the kinetics and to help in the testing of new formulations. The original assay method for the unchanged drug involved isolation on C18 bonded silica AASP cartridges followed by injection of the analyte onto the HPLC column for analysis and quantification [3]. The elution of the drug from the cartridges was effected by an Analytichem Automated Sample Processor (AASP) liquid chromatography module. This preliminary work also found that metabolites II, III and IV were co-extracted along with the parent compound.

The discovery and identification of the glucuronide metabolite (SK&F 94120-MET I) presented a significant analytical challenge as the final aim of the work was to provide an assay for unchanged drug and the four metabolites in one procedure. This was required in order to obtain a complete picture of the absorption and elimination of the drug from a small plasma sample. Additionally a single extraction would require much less work to obtain the same information and less time would be required to process the sample. Consideration was given to the options available to assay the glucuronide metabolite.

Glucuronide metabolites are generally more polar than the parent material from which they are derived and because of this they can be difficult to extract and quantify. Traditionally, analysts have used either acid or the enzyme β D-glucuronidase (E.C. 3.2.1.31.) to hydrolyse a conjugate and then measure the aglycone [4–7]. Acid hydrolysis is non-specific and the authors have preferred to use the enzymatic method. This results in a selective cleavage of the molecule but suffers from several disadvantages:

(1) The enzyme can be inhibited by substances in the sample. Moreover, there is usually no reference material to determine when the hydrolysis is complete and a commercially available conjugate such as phenolphthalein glucuronide is used as a positive control for the enzyme reaction.

(2) The source of the enzyme can result in different activities [8] and care must be taken to ensure that enzyme from the same source is used throughout a study.
(3) Enzymatic hydrolysis is usually carried out overnight and is a long process. Acid hydrolysis is usually quicker due to the higher temperatures employed, however the analyst must be sure that the aglycone is stable under these conditions. In either case compounds or artifacts may result that could interfere with the subsequent measurement of the analyte.
(4) There is much manipulation of sample and solutions which is very time consuming and tedious.
(5) If the parent drug is present in both conjugated and unconjugated forms it requires two assays to measure the two fractions. The first (without hydrolysis) is to determine the unconjugated fraction then the second measures the 'total' (conjugated and unconjugated) material after cleavage. The conjugated fraction is calculated by difference [4].

In summary, hydrolysis methods can be labour intensive and may not always be exact.

To overcome these limitations HPLC post-column hydrolysis can be employed. This is achieved on-line and removes many of the laborious stages of the traditional methods. Elischek [9] has used acid hydrolysis for catecholamine conjugates and Boppana *et al.* have recently used columns packed with glass beads coated with β D-glucuronidase [10] or aryl sulphatase [11] to cleave fenoldopam metabolites. However, these reactor beads are not commercially available and were rejected for this reason. The synthesis of SK&F 94120-MET I enabled us to attempt the analysis of the intact conjugate. This approach has the advantages of simplicity of the analytical procedure, greater speed and specificity.

The majority of literature references has been concerned with the isolation of glucuronide conjugates prior to identification in metabolic studies, rather than quantitative work [12–14]. Analysis has been limited to the characterisation of mainly commercially available glucuronide metabolites by LC–MS [15]; this is an expensive detector and the technique can hardly be said to be routine at present. Kawasaki *et al.* [16] assayed conjugated 17-oxosteroids in urine and serum with isolation on Sep-Pak C18 cartridges followed by elution and derivatisation before instrumental analysis. Whilst this method shows the advantages of liquid–solid sample preparation compared to liquid–liquid extraction [17] the disadvantage is the time required to derivatise the analytes and remove the excess reagent.

Direct injection of urine into a reversed-phase HPLC system to measure the glucuronide and sulphate conjugates of paracetamol (acetaminophen) has been successful [18]. The method used external standardisation to quantify the compounds in the absence of a suitable internal standard; additionally there were no losses related to sample preparation in this case. However, for this approach to be successful, any conjugates must be present in relatively large concentration as well as being separated from endogenous components in the sample matrix.

Presented here is an analytical method for the analysis of SK&F 94120 and its four metabolites in plasma by a single isolation on C18 bonded silica cartridges followed by sequential elution on two AASP HPLC systems. This extends the original work [3] and allows the glucuronide metabolite to be quantified intact without any need for derivatisation.

This assay procedure demonstrates the versatility, power and ease of using bonded silicas for sample preparation.

Experimental

Materials

Analytical grade chemicals were used throughout this study with the following exceptions: methanol and acetonitrile were HPLC grade (Rathburn, Walkerburn, Scotland, UK) and orthophosphoric acid was Aristar grade (B.D.H. Chemicals, Poole, UK). The water used was prepared by deionisation (Milli-Q, Millipore, Molsheim, France) and stored in glass containers for a maximum of one week, then discarded.

All solvents and solutions for HPLC were filtered through *ca* 0.5 μm membrane filters (Millipore type HA and FH for aqueous and organic solvents, respectively). Prior to use, the components of the solvent system were mixed and degassed under reduced pressure, and a helium sparge was maintained through the solvent for the duration of the chromatographic analysis.

Polypropylene centrifuge tubes (1.5 ml) with stoppers were obtained from Sarstedt (Beaumont Leas, Leicestershire, UK). The Vac-Elut manifold and AASP C18 cassettes (reference 707005) for sample preparation (Analytichem International, Harbor City, CA, USA) were purchased from Jones Chromatography, Hengoed, Mid Glamorgan, UK.

Supplies of SK&F 94120, SK&F 94857 [5-(4-propionamidophenyl)-pyrazin-2(1H)-one], the internal standard, and SK&F 94120 metabolites I–IV were obtained from the Department of Synthetic And Isotope Chemistry, Smith Kline and French Research Ltd., Welwyn and were used without further purification.

A standard solution of SK&F 94120 and its four metabolites was prepared by accurately weighing 1.00 mg (1.7 mg of the glucuronide) of each compound into a 500 ml volumetric flask. Water (50 ml) was added and the flask left for 1 min immersed within an ultrasonic water bath (Sonicor Instrument Corporation, Copiague, NY). After this time the flask was removed and made up to volume with water. The internal standard solution was prepared by weighing 1.00 mg SK&F 94857 into a 1000 ml flask containing 500 ml water, which was ultrasonicated for 10 min, removed, and made up to volume with water. Both the stock solutions were stable for at least four weeks provided they were maintained at +4°C when not in use and they were not exposed to direct sunlight.

Plasma samples

Blood from animals that had received SK&F 94120 was withdrawn via a cannula or syringe into heparinised containers, mixed and centrifuged. The plasma obtained was transferred to plain tubes, which were quick frozen over solid carbon dioxide and then kept at −20°C prior to analysis.

Extraction of plasma samples

Plasma samples for the assay of SK&F 94120 and metabolites were first thawed at ambient temperatures and then centrifuged (Varifuge K, Heraeus GmbH, GFR) at 2000 **g** for 10 min to remove the fibrous material that might otherwise block the extraction cartridges. Plasma (100 μl) was transferred to a 1.5 ml polypropylene centrifuge tube then water (100 μl) and internal standard solution (500 μl) were added and mixed by vortex. The internal standard is used to quantify the parent drug and SK&F 94120-MET II, III and IV. In contrast the glucuronide metabolite (SK&F 94120-MET I) is determined by external standardisation.

The AASP C18 cassette was activated by passing methanol (1 ml) and then water

(1 ml) through each cartridge. It is important that the cartridge does not become dry during this activation process otherwise varying analyte recoveries may occur.

Then 300 μl of each diluted plasma sample was transferred to a freshly prepared cartridge and air pressure applied until the reservoir was empty. Water (1.5 ml) adjusted to pH 3 with orthophosphoric acid was then used to wash off any plasma retained in the sorbent bed and any highly polar material adsorbed to the column. The cassette was finally removed from the manifold and transferred to the AASP HPLC systems for automated analysis.

Standard curve

A standard curve was prepared using 100 μl control plasma aliquots that had been spiked with SK&F 94120 and the four metabolites over the concentration range of 0.05–0.50 mg l^{-1}. At the beginning of any study a standard curve was constructed; this consisted of 24 points covering six concentrations; six replicates were assayed for the highest and lowest concentrations and three replicates for each of the four intermediate concentrations. A set of quality control samples was spiked at the same time the samples were received, and stored with them. These quality control samples were processed on each extraction day and any large (>10%) variation from the known value used to adjust the standard curve.

High-performance liquid chromatography

Two chromatographs were set up. Both were identical with the exception of the mobile phases and the instrument settings on the AASP. Details of the AASP have been covered in other publications [3, 17] and will not be repeated here. Each chromatograph consisted of a Model 590 pump (Waters Associates, Milford, MA, USA); the sample extract was introduced into the chromatograph by the AASP LC module with the following settings: System 1: the AASP run time was 6 min, cycle time was 7 min and valve reset switch time was set at 0.3 min; System 2: the respective settings were 8, 9 and 0.5 min.

The analytes were separated by stainless steel columns 300 × 3.9 mm i.d. packed with 10 μm C18-μBondapak (Waters Assoc.) and maintained at 35°C. The column effluents were monitored continuously by Kratos 783 variable wavelength UV detectors set at 280 nm and 0.005 absorbance units full scale. The signals from the detectors were fed into model 301 computing integrators (Laboratory Data Control, Stone, Staffs, UK).

The mobile phase for the first chromatograph (solvent system 1) consisted of 100 ml acetonitrile and 900 ml ammonium acetate buffer (0.01 mol l^{-1}); the pH value of the whole mixture was adjusted to between 4.5 and 5.0 with the addition of 300 μl orthophosphoric acid. This was pumped through the system at 2.0 ml min^{-1} and under these conditions the retention time of SK&F 94120-MET I was *ca* 4 min.

The solvent system used in the second chromatograph consisted of 200 ml acetonitrile, 800 ml ammonium acetate buffer (0.01 mol l^{-1}) and 300 μl orthophosphoric acid; the pH value of the mobile phase was between 4.5 and 5.0. At a flow rate of 2 ml min^{-1} the approximate retention times of SK&F 94120, MET III, SK&F 94857 (the internal standard), MET IV and MET II were 3, 3.5, 4.5, 6 and 7 min, respectively.

The column in either system was allowed to equilibrate with the respective mobile phase for at least 1 h before analysis. After use the column was flushed with acetonitrile–water (20:80, v/v) for a minimum of 30 min. The mobile phase was not recycled because of a build up of analytes in the solvent which affected the baseline

stability of the detector. Prior to and after analysis a blank AASP cassette (i.e. one which has no sorbent in the cartridges), was processed through the AASP with the cycle time set to 1 min in order to flush the fluid pathways of the AASP and valve.

Quantification

Chromatograph 1 measured the concentrations of SK&F 94120 MET I in the sample by the external standard method whereby the peak area measurements were plotted against known concentrations of the glucuronide to obtain a regression line. The co-ordinates of this line were then used to calculate the concentration of SK&F 94120 MET I in the samples.

In contrast chromatograph 2 used internal standardisation; here the peak areas of the analytes and the internal standard were measured by the integrator. Individual analyte/internal standard ratios were plotted against the respective analyte concentration of the standards and a regression line obtained. This was used to calculate the unknown compound concentration in the sample.

All standard curves were rectilinear over the concentration range 0.05–0.5 mg l^{-1}; the slopes and intercepts of the respective standard curves are shown in Table 1.

Table 1
Slope and intercept values from the calibration curves of SK&F 94120 and its four metabolites

	MET I*	SK&F 94120	MET II	MET III	MET IV
r^2	9.6	98	99	100	99
Slope	210507	4.20	2.87	4.19	2.35
Intercept	3455	0.18	−0.04	−0.05	−0.02

* Calculated by external standardisation using peak area measurement.

Results and Discussion

Analytical strategy for the assay of SK&F 94120 and its metabolites

Having decided to measure the glucuronide metabolite intact, the feasibility of including the metabolite determination in the established assay procedure was assessed. The ability of the C18 AASP cassette to extract this analyte and retain it during the washing stage was investigated. Under the conditions of the standard assay, extraction of the glucuronide from plasma by the bonded phase was quantitative but increasing volumes of water, used to wash the cartridge of remaining plasma and polar material, reduced the recovery by 50% (Fig. 2). Consideration was given to the problem and it was decided that a lower pH value wash solution should give a higher recovery as the carboxylic acid moiety of the molecule would become unionised and hence less hydrophilic. This is shown in Fig. 2 where water adjusted to pH 3 with orthophosphoric acid did not lead to loss of the analyte in contrast to the normal water wash (pH 6). Thus, a simple modification to the existing assay gave quantitative extraction of this compound with no effect on the recoveries of the drug and remaining metabolites.

A chromatogram obtained using the original mobile phase (20% acetonitrile, 80% ammonium acetate buffer) for parent drug [3] is shown in Fig. 3. The glucuronide is barely retained on the column, eluting just behind the void volume. This chromatogram is of standard solutions taken through the assay; however, co-extracted components from

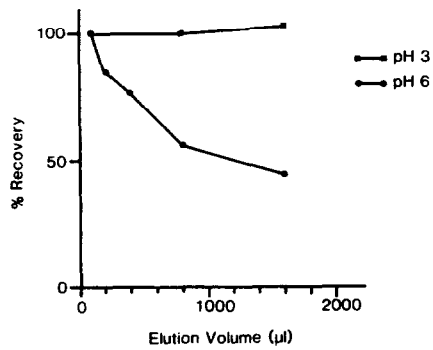

Figure 2
Effect of water pH on the recovery of SK&F 94120-O-glucuronide (metabolite I) from a C18 AASP cassette.

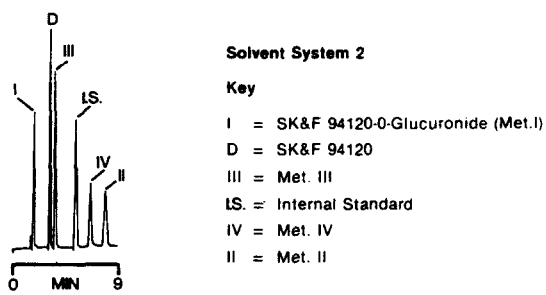

Figure 3
HPLC–UV Chromatogram of SK&F 94120 and metabolite reference standards.

the biological matrix produce interfering peaks that prevent quantification of this metabolite and render the assay impracticable.

Thus, a new strategy was necessary to resolve the glucuronide from the solvent front and yet permit the achievement of a single sample preparation step to measure the parent drug and the four metabolites.

It was decided that sequential elutions of the same AASP cassette would be the best approach. This should selectively remove the glucuronide metabolite from the solid phase for measurement whilst the parent drug and the remaining three metabolites were not eluted. The cassette would then be transferred to a second chromatograph for measurement of the parent drug and metabolites II, III and IV. This strategy is shown in Fig. 4.

For this strategy to work, the glucuronide should be selectively eluted from the solid phase without affecting the retention of the other analytes. Additionally, the second chromatograph should elute the remaining analytes without excessive peak broadening.

Solvent system 1

Increasing volumes (from 220–2200 µl) of solvent system 1 (10% acetonitrile) were passed through a cartridge containing 50 ng each of the SK&F 94120 and its four metabolites. After this the cartridge was eluted into the chromatograph using solvent system 1 and valve reset facility set to 1 min. The percentage recovery of the analytes was

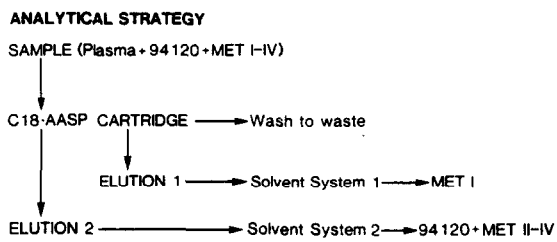

Figure 4
Analytical strategy for the measurement of SK&F 94120 and metabolites I–IV in plasma.

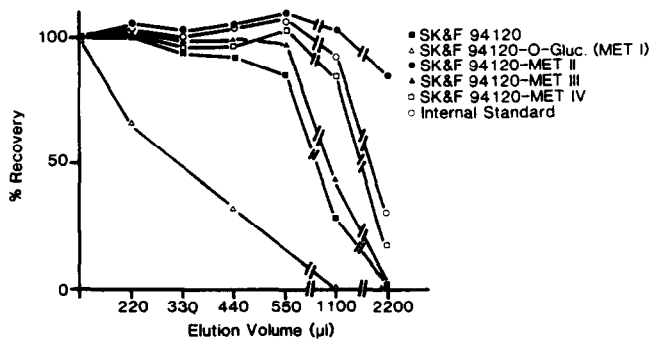

Figure 5
Effect of cartridge wash volume (solvent system 1) on the recovery of SK&F 94120 and metabolites I–IV.

calculated from the peak area measurements compared to a calibration standard injected directly into the chromatograph. This data is shown in Fig. 5.

It can be seen that the glucuronide metabolite elutes before the remaining compounds, however the profile is not so steep as to selectively elute the former without affecting the recovery of one of the other analytes. Increasing the pH value of the mobile phase up to 7 (to ensure complete ionisation of the carboxylic acid) had no effect on the elution profile: this was in marked contrast to the use of pH adjustment to isolate the compound from plasma. This phenomenon, though interesting, remains unexplained.

The valve reset facility was therefore used to control the elution of MET 1 from the cartridge. At a flow rate of 2 ml min^{-1} and a valve reset time of 0.3 min, 600 µl of mobile phase flowed through the AASP cassette which resulted in an 85% recovery of the glucuronide but only 10–15% loss of SK&F 94120.

Solvent system 2

A similar experiment was conducted to optimise the valve reset time on the remaining analytes. SK&F 94120 and metabolites II, III and IV (50 ng) were extracted onto a C18 cartridge and eluted with different volumes of solvent system 2. The results are shown in Fig. 6. These indicate that after elution with 1000 µl of mobile phase there is quantitative recovery of the analytes remaining on the cartridge.

Chromatography

Typical chromatograms observed after the elution of analytes from the AASP extraction cartridges are shown in Figs 7 and 8. Figure 7 shows typical chromatograms for

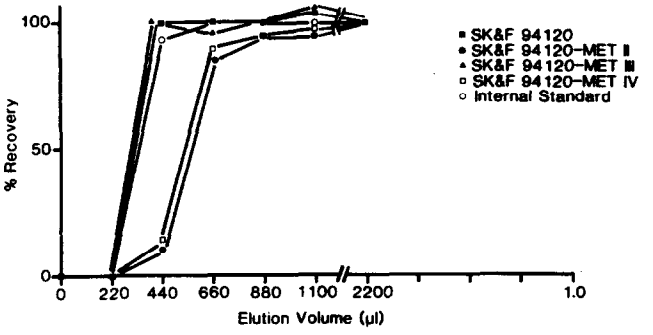

Figure 6
Effect of valve reset time on the recovery of SK&F 94120 and metabolites II–IV.

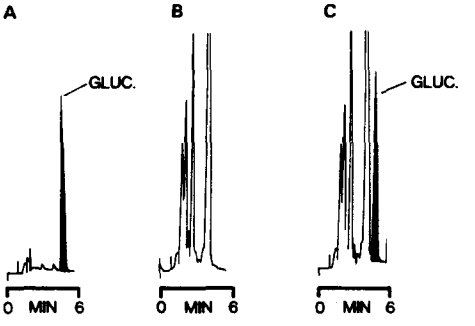

Figure 7
Typical chromatograms in solvent system 1. (a) SK&F 94120-MET 1 reference compound; (b) extract of control dog plasma; (c) extract of control dog plasma spiked with MET 1 (0.25 mg l^{-1}).

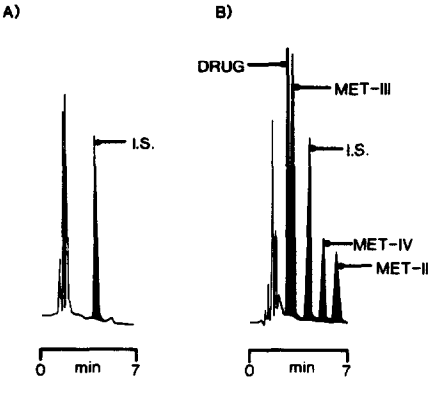

Figure 8
Typical chromatograms in solvent system 2. (a) Extract of control plasma containing internal standard; (b) extract of control plasma containing internal standard spiked with SK&F 94120 and metabolites II–IV.

the standard solution, control dog plasma and control plasma spiked with the glucuronide metabolite taken through the procedure and eluted in solvent system 1. Figure 8 shows the elution of the remaining analytes from the cartridge in solvent system 2. In both cases the peak shape of the analytes is good and there is adequate resolution for accurate quantification.

No endogenous compounds with retention times corresponding to either SK&F 94120, its internal standard (SK&F 94857), or the four metabolites have been observed in extracts of samples chromatographed in the two solvent systems used in this assay. This includes the predose samples from cynomolgus monkey, rats, mice and dogs receiving the compound as well as the control plasma from these species used to validate the assay.

Precision and accuracy

The precision and accuracy of the assay were assessed by spiking and assaying replicate samples of plasma containing known concentrations of SK&F 94120 and its four metabolites.

The results are shown in Table 2; the precision as measured by the coefficient of variation (CV), is shown over the range 0.125–0.500 mg l^{-1}. The assay precision is good, being 10% or less at the concentrations studied.

The accuracy of the assay (measured as bias) is generally within 10%. The large value of 12% at 0.25 mg l^{-1} SK&F 94120 is due to one outlier, otherwise the accuracy is good.

These figures are greater than quoted for the original work for the parent drug [3], however they are acceptable given the greater amount of information derived from the assay.

Table 2
Precision and accuracy for the analysis of SK&F 94120 and metabolites I–IV in dog plasma

PRECISION

Conc. (mg/l)	Precision (±CV%)					
	n	SK&F 94120	MET I	MET II	MET III	MET IV
0.125	5	5	5	8	4	2
0.250	5	10	5	9	7	5
0.375	5	7	5	6	6	4
0.500	5	1	6	7	5	4

ACCURACY

Conc. (mg/l)	Bias (% error)					
	n	SK&F 94120	MET I	MET II	MET III	MET IV
0.125	5	−2	+6	<+1	−6	+6
0.250	5	−12	−4	−6	+4	−8
0.375	5	−1	0	−5	−1	−7
0.500	5	+3	<+1	+4	0	+4

n = number of determinations.

Conclusions

The assay presented here shows the power attainable with liquid–solid extraction techniques. The assay is robust, quick, efficient and is capable of a large throughput of samples which makes it ideal for a busy pharmaceutical laboratory. The assay has the potential for complete automation and this possibility is under investigation.

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