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ANALYSIS OF 5-(4-ACETAMIDOPHENYL)PYRAZIN-2(1H)-ONE (SK&F 94120) IN PLASMA WITH AN ANALYTICHEM AUTOMATED SAMPLE PROCESSOR LIQUID CHROMATOGRAPHY MODULE

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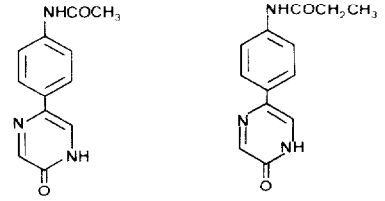
SUMMARY

A selective and specific assay for 5-(4-acetamidophenyl)pyrazin-2(1H)-one (SK&F 94120), a novel inotropic agent, has been developed. The method incorporates a liquid–solid extraction step with a C_{18} Analytichem automated sample processor (AASP) cassette, which consists of ten miniature extraction columns. The cassette is then loaded into the AASP auto injector, ready for automated liquid chromatography with UV detection. The AASP consists of a high-pressure sealing chamber which encapsulates each column. The high-performance liquid chromatographic mobile phase is directed through the chamber, and the analytes are eluted onto the analytical column for subsequent separation and measurement.

The assay is sufficiently accurate and precise to determine SK&F 94120 at concentrations as low as 0.5 mg/l. The mean coefficient of variation for the concentration range 0.5–10.0 mg/l was 2% with a bias of ± 1%. The assay has been used for pharmacokinetic and bioavailability studies in several species, including rat, dog, and cynomolgus monkey.

INTRODUCTION

5-(4-Acetamidophenyl)pyrazin-2(1H)-one (SK&F 94120, Fig. 1) is a novel, potent, orally active, positive inotropic agent with vasodilator activity, considered useful for the treatment of congestive heart failure[1].



SK&F 94120 **SK&F 94857**

Fig. 1. Structures of SK&F 94120 and SK&F 94857 (internal standard).

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Developments in bonded-silica chemistry, used to improve high-performance liquid chromatographic (HPLC) columns, has led to the introduction of the modern manual methods of liquid–solid extraction with small disposable cartridges: Sep-Pak (Waters Assoc., Milford, MA, U.S.A.) and Bond Elut (Analytichem International, Harbor City, CA, U.S.A.)[2]. This type of extraction retains selectivity, improves the speed and ease of sample preparation, but does not require expensive, high-purity solvents and completely eliminates the formation of emulsions. The sample is applied to the top of the cartridge and the flow through it is usually achieved by applying vacuum to the bottom of the column. Batch analysis is possible with commercially available manifolds that contain eight or ten cartridges.

The use of a liquid–solid extraction procedure offers many advantages over more conventional liquid–liquid extractions[3]. These include the complete elimination of emulsion formation, the use of small plasma volumes and a reduction in the volume of expensive and hazardous solvents. The availability of a wide range of chemically bonded phases allows for selective extraction of analytes of interest from the complex biological matrix[4–6].

In our initial work on an assay for SK&F 94120 in plasma we used C_{18} Bond Elut cartridges, but the availability of the Analytichem automated sample processor (AASP), a semi-automatic apparatus, combining extraction and HPLC analysis, focussed our attention on this instrument as a means of improving the assay efficiency, reproducibility, and sensitivity. We present here a method for the assay of SK&F 94120 in plasma by liquid–solid extraction with a C_{18} silica column and subsequent analysis with the AASP liquid chromatography module.

EXPERIMENTAL

Materials

Analytical-grade chemicals were used throughout this study with the following exceptions: methanol and acetonitrile were HPLC grade (Rathburn, Walkerburn, U.K.), and orthophosphoric acid was Aristar grade (B.D.H. Chemicals, Poole, U.K.). The water used was prepared by deionisation (Milli-Q, Millipore, Molsheim, France) and stored in glass containers.

All solvents and solutions for HPLC were filtered through 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. A flow of helium was maintained through the solvent reservoir for the duration of the chromatographic analysis.

The polypropylene centrifuge tubes (1.5 ml) with stoppers were obtained from Sarstedt (Beaumont Leys, U.K.). The Vac-Elut manifold and AASP C_{18} cassettes (Analytichem International) were purchased from Jones Chromatography (Llanbradach, U.K.).

Supplies of SK&F 94120 and the internal standard 5-(4-propionamidophenyl)pyrazin-2(1H)-one (SK&F 94857, Fig. 1) were obtained from the Department of Synthetic & Isotope Chemistry, Smith Kline and French Research, Welwyn, and were used without further purification. Standard solutions of both compounds were prepared by accurately weighing 1.00 mg of each compound into 100- and 1000-ml volumetric flasks, respectively.

To the flask containing SK&F 94120 was added 50 ml water and the flask and its contents were left for 1 min immersed within an ultrasonic water bath (Sonicor Instrument Corporation, Copiague, NY, U.S.A.). After this time the flask was removed and the volume made up to 100 ml with water. To the flask containing the internal standard was added 500 ml water, after which it was ultrasonicated for 10 min, removed, and made up to volume with water. Both 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 been treated with SK&F 94120 was withdrawn via a cannula or syringe into heparinised containers, mixed, and centrifuged. The plasma was transferred to plain tubes, which were quick-frozen over solid carbon dioxide and then kept at −20°C pending analysis.

AASP

The AASP liquid chromatography module is a microprocessor-controlled instrument, specifically designed to integrate extraction on bonded-phase columns with automated syringeless injection of the extract into a high-performance liquid chromatograph. Samples are prepared off-line with a cassette of ten miniature extraction columns (Fig. 2). These are similar in design to Bond Elut columns, but fit into a modified Vac-Elut manifold (Analytichem International). A sample reservoir is then placed on top, into which the sample is dispensed, followed by the application of pressure (nitrogen or air) in order to drive it through the sorbent bed.

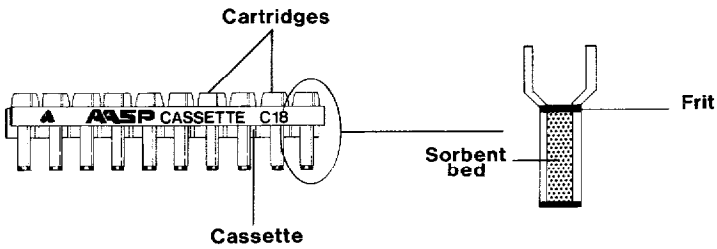


Fig. 2. AASP cassette.

After the samples have been applied, the columns may be washed to remove any remaining traces of plasma or undesirable material. In this respect, AASP sample preparation is essentially similar to the Bond Elut method. However, elution is not carried out in the normal manner. The prepared cassettes, containing the analytes, are loaded into the AASP auto-injector. Up to ten cassettes (*i.e.* 100 samples) can be loaded at any one time.

The AASP auto-injector consists of two hoppers: an infeed, containing the cassettes of sample extracts to be analysed, and a waste, where the used cassettes are placed by the instrument pending disposal. Connecting the two hoppers is the cassette feeding mechanism, which takes the next cassette and places each extraction column into a high-pressure sealing chamber (Fig. 3), which encapsulates each column. Elu-

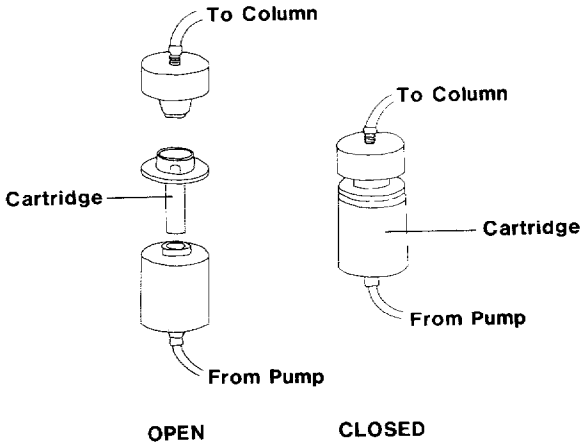


Fig. 3. Schematic drawing of AASP compression chamber in open and closed positions.

tion of the analytes is effected by switching the solvent flow from the HPLC pump through this chamber and onto the analytical column. The fluid pathway is determined by a pneumatically activated Valco 10-port injection valve (Valco, Houston, TX, U.S.A.), controlled by the AASP microprocessor (Fig. 4). This valve can also be reset after a predetermined time period. Thus, the analytes are selectively eluted from the extraction cartridges, leaving unwanted endogenous compounds behind. These compounds, if eluted onto the analytical column, would greatly increase the analysis time.

The instrument has three operational modes: "Manual" for processing individual samples; "Remote" for automatic analysis, controlled from an external device; and "Auto" for automatic analysis, controlled by the AASP itself. In addition to the off-line column washes, a purge facility is available. Prior to injection, the cartridge can be flushed with aliquots of a suitable solvent from a separate reservoir (Fig. 4). The eluent in this instance is sent to waste.

Extraction of plasma samples

Plasma samples for the determination of SK&F 94120 were first thawed at ambient temperatures and then centrifuged (Varifuge K, Heraeus Christ GmbH, F.R.G.), at 2000 *g* for 10 min to remove fibrous material that might otherwise block the extraction columns. A volume of 100 μl of plasma was transferred to a 1.5-ml polypropylene centrifuge tube, and 100 μl water and 500 μl of the internal standard solution were added and mixed by a Vortex mixer. If there was less than the nominal volume of plasma, then this was made up by the addition of a suitable volume of control plasma.

The AASP C_{18} cassette was activated to extract the analytes by passing 1 ml methanol and then 1 ml water through each column. It is important that the column does not become dry during this activation process, otherwise varying recoveries may ensue.

Then, 300 μl of each plasma sample was transferred to a newly prepared car-

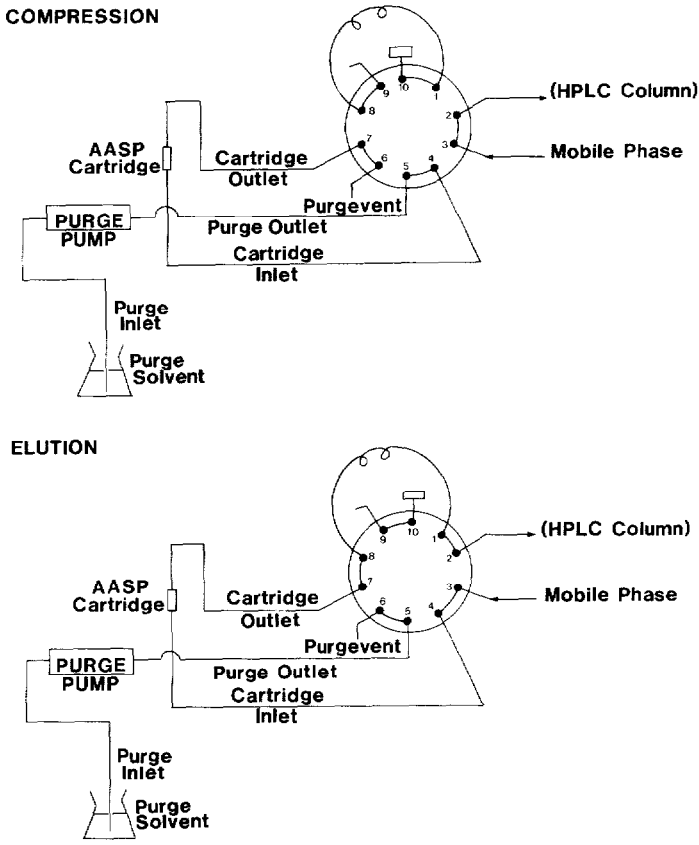


Fig. 4. AASP fluid pathway during compression and elution cycles.

tridge, and air was applied until the reservoir was empty. Water (1 ml) was then used to wash off any plasma retained in the cartridge bed and any highly polar material adsorbed to the column. The cassette was removed from the manifold and transferred to the AASP for automated HPLC analysis.

HPLC

The chromatograph consisted of a Model 590 (Waters Assoc.) pump, and the sample extract was introduced into the system by the AASP liquid chromatography module. The analytes were separated on a stainless-steel column, 300 × 3.9 mm I.D., packed with 10 μm μBondapak (Waters Assoc.) and maintained at 35°C. The column effluent was monitored by a Model 441 fixed-wavelength UV detector (Waters Assoc.) equipped with a mercury lamp and 280 nm filter and set at 0.005 a.u.f.s. The signal from the detector was fed into a Model 301 computing integrator (Laboratory Data Control, Stone, U.K.).

The solvent system consisted of acetonitrile–0.01 *M* ammonium acetate buffer–orthophosphoric acid (20:80:0.03). The pH value of the mobile phase should be between 4.5 and 5.0. The column was allowed to equilibrate with the mobile phase

for at least 1 h before analysis. After use, the column was flushed with acetonitrile–water (40:60, v/v) for a minimum of 30 min. The recycling of mobile phase was not undertaken, due to the 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.

At a flow-rate of 2 ml/min, the approximate retention times of SK&F 94120 and SK&F 94857 were 3 and 4.5 min, respectively.

Quantification

The calibration curve for SK&F 94120 in plasma was constructed from replicate samples at six concentrations over the range 0.50–10.0 mg/l. The ratio of the drug peak height to internal standard peak height was plotted against the known concentrations of drug in the standards. A linear regression was obtained, and unknown concentrations were read directly from the standard curve. The curve was found to be rectilinear over the range studied. The slope, intercept, and correlation coefficient were 0.350, 0.002, and 0.999, respectively.

A set of quality control samples, spiked at the same time the samples were received and stored with them, was processed on each extraction day and any day-to-day variation was used to adjust the standard curve.

RESULTS AND DISCUSSION

Chromatography

Typical chromatograms observed after the elution of analytes from the AASP extraction cartridges are shown in Fig. 5. No endogenous compounds with retention times corresponding to either SK&F 94120 or SK&F 94857 have been encountered in the predose samples from animal species cynomolgus monkey, rats, mice, and dogs nor in the control plasma from these species, used to validate the assay. In the course

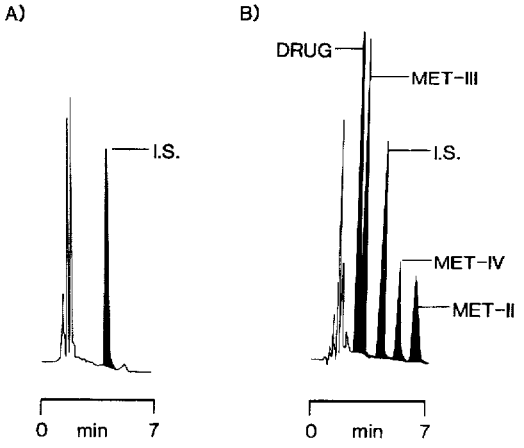
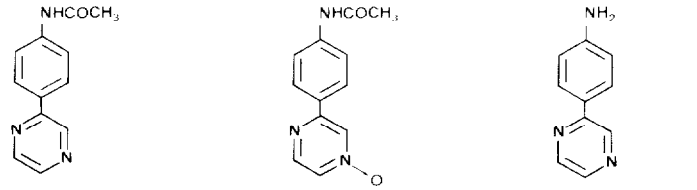


Fig. 5. Typical chromatograms of extracts of control rat plasma spiked with (A) internal standard (I.S.) and (B) internal standard (I.S.), drug and three metabolites (MET-II, MET-III and MET-IV).

of studies involving the administration of SK&F 94120, additional peaks were observed that corresponded to three metabolites. These have been characterised by mass spectrometry and nuclear magnetic resonance spectrometry and shown to be 2(4-acetamidophenyl)pyrazine (94120-MET-II), 3-(4-acetamidophenyl)pyrazine-1-oxide (94120-MET-III), and 2-(4-aminophenyl)pyrazine (94120-MET-IV) (Fig. 6).



94120-MET-II 94120-MET-III 94120-MET-IV

Fig. 6. Structures of circulating plasma metabolites of SK&F 94120.

All three metabolites were well separated from the parent drug, as shown in the chromatograms in Fig. 5. A major metabolite of SK&F 94120 in rat, dog, and monkey has been tentatively identified as a glucuronide, conjugated through the pyrazine oxygen. The assay reported in this paper has the potential to measure this conjugate intact, and this possibility is under investigation.

Assay validation

Recovery. The recovery of SK&F 94120 and SK&F 94857 was quantitative over the range studied. This was determined by comparison of the peak area measurements of standards which had been taken through the extraction procedure with those injected directly onto the chromatograph.

Precision and bias. The precision and bias of the assay were assessed over two separate days by spiking and assaying replicate samples of plasma, containing known concentrations of SK&F 94120. A summary of the results is given in Table I. The precision, expressed as the coefficient of variation, and the bias, expressed as percentage error, were excellent over the range 0.50–10.0 mg/l.

TABLE I

PRECISION AND BIAS OF AASP ASSAY FOR SK&F 94120 IN DOG PLASMA

Bias calculated as $\left(\frac{\text{mean calculated concentration} - \text{actual concentration}}{\text{actual concentration}}\right) \cdot 100\%$.

Concentration of SK&F 94120 (mg/l)	*Precision (coefficient of variation, %)*	*Bias (error, %)*	*Number of determinations*
0.5	3	−1	12
1.0	<1	<−1	6
2.5	2	<−1	6
5.0	2	+1	6
7.5	2	<+1	6
10.0	2	<−1	12

Employment of the AASP (liquid chromatography module) resulted in a 50% saving in sample preparation time by virtue of the reduction in liquid handling and there was also a marked improvement in precision of the assay as highlighted in Table II, compared with the Bond Elut method.

TABLE II

COMPARISON OF PRECISION FOR MANUAL AND AASP ASSAYS OF SK&F 94120 IN DOG PLASMA

Concentration of SK&F 94120 (mg/l)	*Precision (coefficient of variation, %)*	
	Bond Elut	*AASP*
0.10	14	2
1.00	11	2
5.00	19	2

Stability of SK&F 94120 in plasma. Plasma samples were spiked with SK&F 94120 at 1- or 10-mg/l levels and stored at −20°C for up to ten weeks. The stability of SK&F 94120 was assessed by comparing the results obtained from assaying the stored samples with those for freshly prepared standards. The results indicated that there was no degradation of drug under these conditions over the time period investigated.

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REFERENCES

1 W. J. Coates, R. J. Eden, J. C. Emmett, R. W. Gristwood, D. A. A. Owen, R. A. Slater, E. M. Taylor and B. H. Warrington, *Br. J. Pharmacol.*, 84 (1985) 22p.
2 M. R. Harkey and M. L. Stolowitz, *Adv. Anal. Toxicol.*, 1 (1984) 255.
3 R. D. McDowall, J. C. Pearce and G. S. Murkitt, *J. Biomed. Pharm. Anal.*, in press.
4 T. J. Good and J. S. Andrews, *J. Chromatogr. Sci.*, 19 (1981) 562.
5 R. N. Gupta, R. B. Haynes, A. G. Logan, L. A. Macdonald, R. Pickersgill and C. Achber, *Clin. Chem.*, 29(6) (1983) 1085.
6 M. A. Elsohly, H. N. Elsohly, A. B. Jones, P. A. Dimson and K. E. Wells, *J. Anal. Toxicol.*, (1983) 262.