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Two systems for the automated analysis of drugs in biological fluids using high-performance liquid chromatography[a]

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SUMMARY

This paper describes two fully automated assays. One for zaprinast, a cGMP specific phosphodiesterase inhibitor, which uses the Gilson-Advanced Automated Sample Processor combination, and the other for an H^+/K^+ ATPase inhibitor and its sulphone metabolite, which uses direct injection. Both assays were developed to support pharmacokinetic studies at therapeutic doses in small animals as well as in man. Plasma or serum (20–200 μl) is placed directly into an autosampler and all subsequent manipulations are performed mechanically.

INTRODUCTION

The analysis of drugs from biological fluids involves their separation from a biological matrix (e.g. blood, plasma, urine or bile). The initial sample presented to the analyst is therefore complex, containing high-molecular-mass proteins, which are incompatible with sorbents derived from silica, and compounds of greatly different polarity coming, for example, from the food chain. Traditionally the drug is removed from the sample matrix using selected solvents and back-extracted to separate acids from bases. However, today the emphasis is on development of general methods to extract both drug and metabolites simultaneously, and for similar compounds in a chemical series of

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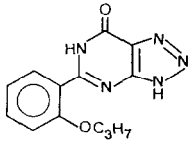
candidate drugs. Specificity is achieved by high-performance liquid chromatographic (HPLC) separation, derivatisation and specific detectors such as electrochemical detectors. The sensitivity of the method should be sufficient that it can be used to define the clinical pharmacokinetics of the drug, and it is often required that the assay should support pharmacokinetic studies at therapeutic doses in small animals when only a very small plasma sample (20 μl) may be available.

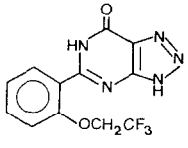
Solid-phase extraction columns are particularly well suited to these requirements [1]. Small columns packed with chemically bonded silica derived from HPLC column technology provide a convenient means of extracting the analyte and introducing the total extract onto the HPLC analytical column. Solid-phase extraction is simple, and sensitivity is attained because there is no sample loss through transfer. In fact the sample handling and HPLC separation stages of an assay are now so similar in their operation that they can be built into a single analytical system, providing the analyst with a fully automated assay. When sample preparation and HPLC are performed together, the limitation on the number of samples that can be assayed in a day shifts from human constraints placed on it by a technician to the reliability of the equipment and the HPLC run-time. Today, advances in sample handling methods and the now routine use of fully automated assays have achieved this shift.

Two systems which the authors use to provide full automation to sample handling are the Advanced Automatic Sample Processor (AASP, Varian Assoc., Walton-on-Thames, U.K.) in combination with a Gilson 222/401 autosampler with dilutor (Anachem, Luton, U.K.) [2] and direct injection which was first described by Roth et al. [3]. Both these systems are dedicated sample preparation units developed specifically for routine assays; they are relatively inexpensive, highly productive and can also be adapted easily from method to method.

This paper briefly describes two fully automated assays used in the authors

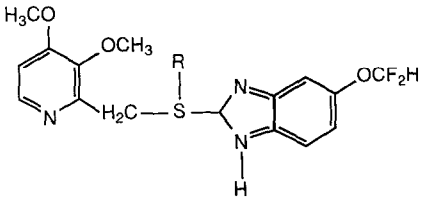


I



Internal standard

Fig. 1. Structures of I and internal standard.



R = 0 : II
R = 0,0 : II-A

Fig. 2. Structures of II and II-A.

laboratory; one for zaprinast (I, Fig. 1), a cGMP specific phosphodiesterase inhibitor [4,5], which uses the Gilson–AASP combination, and the other for 5-difluoromethoxy-2-[(3,4-dimethoxy-2-pyridyl)-methylsulphinyl]-1H-benzimidazole (II, Fig. 2), an H^+/K^+ ATPase inhibitor, and its sulphone metabolite II-A (Fig. 2), which uses direct injection [6].

EXPERIMENTAL

Materials

C_2-AASP cassettes containing 50 mg of sorbent were manufactured by Analytichem International (Harbor City, CA, U.S.A.) and supplied by Jones Chromatography (Llandbrdach, U.K.). LiChroprep RP-2, 20–40 μm (Merck) 9was supplied by BDH (Poole, U.K.), and pre-columns for direct injection (10 mm × 4.6 mm) were obtained from Bischoff (Leonberg, F.R.G).

Standard compounds were synthesised by Smith Kline and French Research (Welwyn, U.K.) and were > 99% pure by HPLC analysis. Reagents were supplied by either May and Baker (Dagenham, U.K.) or BDH and were AnalaR grade unless otherwise stated.

HPLC of I in plasma using the Gilson–AASP combination

Apparatus. The HPLC equipment consisted of a Waters Assoc. Model 590 pump (Millipore, Harrow, U.K.), a Kratos Spectroflow 783 or 757 detector (Severn Analytical, Shefford, U.K.) monitoring at 275 nm and an AASP manufactured by Analytichem International and supplied by Jones Chromatography. Automated sample preparation was performed by a Gilson 222 autosampler and 401 dilutor (Anachem), linked directly to the AASP. Chromatographic recordings and integrations were performed on an LDC 301 recording integrator (Laboratory Data Control, Stone, U.K.).

The reversed-phase analytical column was prepacked with Ultrasphere™ C_8, particle size 5 μm (Altex; 150 mm × 4.6 mm I.D.; supplied by Beckman-RIIC, High Wycombe, U.K.), and it was maintained at 40°C using a Dupont 8800 column oven.

Mobile phase. Acetic acid (50 mmol l^{-1}) and ammonium acetate (50 mmol l^{-1}) were mixed together to pH 4.0. A volume of this solution (650 ml) was then combined with acetonitrile (350 ml) to make the mobile phase.

Before use, the acetonitrile was filtered through 0.5-μm membrane filters, type FH, and the acetate buffer through 0.45-μm membrane filters, type HA (Millipore). The flow-rate was 1 ml min^{-1} and any dissolved gases were removed by sparging with helium before and during use.

Sample preparation prior to automation. The initial preparation of samples, and spiking of standards or quality controls, was performed manually. A mixture of methanol–water (125 μl, 4:96) was added to plasma (20 μl) in a 5-ml plastic Rohren tube. The mixture was added so that the volume extracted would be consistent with that of the calibration standards, which were prepared by adding a solution of I in methanol–water (4:96) to drug-free plasma. The tubes were then placed in the Gilson 222 sample rack ready for analysis.

Automated sample extraction. The Gilson 222/401 was first used to add acidified internal standard solution, 5-([2-(2,2,2-trifluoroethoxy)-phenyl]triazolo[4,5-*d*]pyrimidin-7-one; 1 ml) to the plasma (20 μl) in the Gilson sample rack. The Gilson was connected to the purge port of the AASP switching valve (port 5; Fig. 3), so that the solid phase was activated on-line, sample added and washed, and then automatically eluted onto the analytical column. The Gilson 401 dilutor, in conjunction with the 222, was used to draw the following liquids into a holding loop in reverse order; methanol (1 ml) and water (1 ml) required to activate the solid phase, plasma and internal standard mixture (400 μl) and acetate buffer (0.1 *M*, pH 8.0, 300 μl) used to wash the cartridge. The liquids were each separated by a segment of air (Fig. 3). The liquid 'train' was then passed via the injection port of the Gilson 222, through the AASP cartridge in situ in the AASP. After sample extraction, the mobile

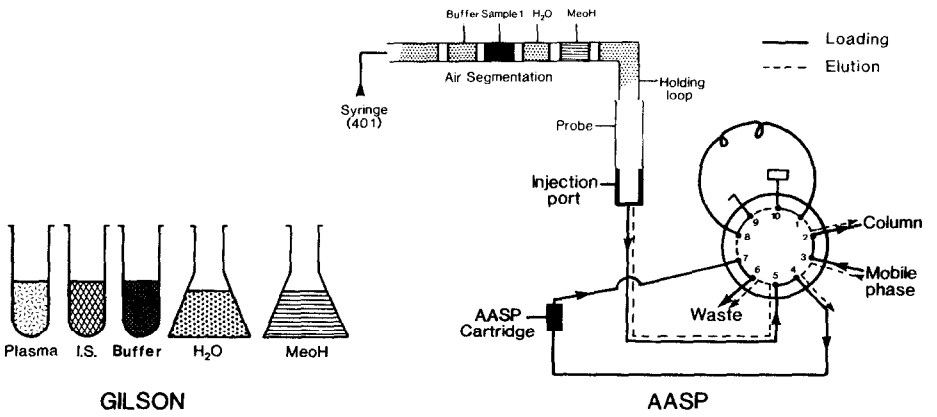


Fig. 3. Schematic of Gilson–AASP automated system.

phase was switched through the cartridge, eluting the drug, internal standard and any other retained compounds, directly onto the analytical column.

Software for use with the Gilson–AASP system was stored on disk. The programs were loaded into the Gilson memory using an Apple IIe or IBM-PC compatible microcomputer. Once loaded, the programs were stored for use in individual files.

The Gilson 222 assumed responsibility as the master controller and communicated with the AASP via a 12-V interface relay. In this way the autosampler could remotely start the AASP and advance the cassette at a predetermined time. After the elution of each sample from the AASP cartridge, the AASP was programmed to reset to the load position after 0.6 min.

HPLC of II and II-A using direct injection

Apparatus. The HPLC equipment was the same as that for I except a Waters WISP 710B autosampler (Millipore) was used to replace the Gilson and an automated gradient controller (Millipore) was used for gradient control (Fig. 4).

A pre-column (10 mm×4.6 mm I.D.) packed with LiChroprep RP-2, 25–40 μm, connected across a six-port Rheodyne 7010 (Millipore), was used for extraction, the detector was set to monitor at 290 nm, and a Chromatography Laboratory Automation System (CLAS; Perkin-Elmer, Beaconsfield, U.K.) was used for integration.

The Rheodyne 7010 was activated by a 12-V signal timed and provided by the integration method. A specially designed relay system was developed within the authors' laboratory to convert a contact closure provided by an integrator into a 12-V signal required by the Rheodyne.

The reversed-phase analytical column (125 mm×4.6 mm I.D.) was pre-

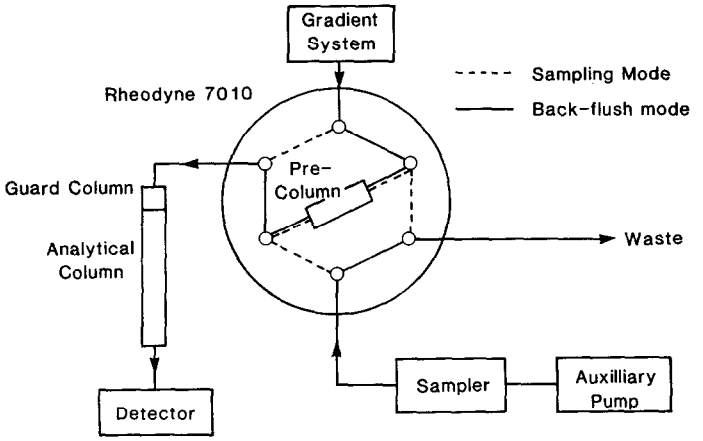


Fig. 4. Schematic of direct injection.

packed with Hypersil RP-18, 5 μm (Jones Chromatography), fitted with a guard column (12 mm × 4.6 mm I.D.) containing the same Hypersil phase.

Extraction eluent. The extraction eluent was made by mixing sodium acetate buffer (50 mmol l^{-1}, pH 5) and acetonitrile (90:10, v/v) and metered by the auxiliary pump at a flow-rate of 1.5 ml min^{-1}. Any dissolved gases were removed by sparging with helium before and during use.

Analytical column mobile phase. A gradient using methanol and ammonium phosphate (10 mmol l^{-1}, pH 6.5) was used as follows: initial conditions, methanol–buffer (43:57, v/v); 0–2 min methanol increasing linearly to 83:17 (v/v); 19–21 min flushing period using 100% methanol; 21–28 min equilibrium period using initial conditions.

Before use, the methanol was filtered through 0.5-μm membrane filters, type FH, and the phosphate buffer through 0.45-μm membrane filters, type HA (Millipore). The flow-rate was 1 ml min^{-1} and any dissolved gases were removed by sparging with helium before and during use.

Sample preparation prior to automation. Prior to analysis, plasma samples were centrifuged for 10 min at approximately 2000 *g* to remove solid material. This method did not use an internal standard and all other manipulations were done on-line.

Direct injection. The sample was injected onto the pre-column in a flow of extraction eluent. After 2 min the flow of solvent was switched using a high-pressure switching valve so that the gradient passed through the pre-column and the analytical column (Fig. 4). The pre-column was dry packed and meshes rather than frits were used to avoid blockage. The column was 'primed' two or three times with control sample before use. The priming may serve to deactivate the silica backbone of the column and avoid a dual mechanism of retention in which the analyte interacts with the residual silanols as well as being retained by interaction with bonded alkyl chains on the stationary phase surface [7,8]. With this system, the pre-column was used for approximately 150 injections of plasma (100 μl). Deterioration of the pre-column was indicated by an increase in back-pressure. The pre-column was flushed separately from the analytical column with the extraction eluent and allowed to equilibrate at the loading conditions between assays because a gradient was used.

RESULTS AND DISCUSSION

The analysis of I only involved sample pretreatment in the spiking of calibration standards. The recovery of I and its internal standard was greater than 90% in the range 1–25 μg ml^{-1}. The precision (coefficient of variation, c.v.) was 13% at 1 μg ml^{-1} and better than 10% at higher concentrations. Chromatograms to blank plasma and a spiked plasma sample containing 10 μg ml^{-1} I are shown in Fig. 5.

Under the conditions described for the assay of II and II-A the recovery of

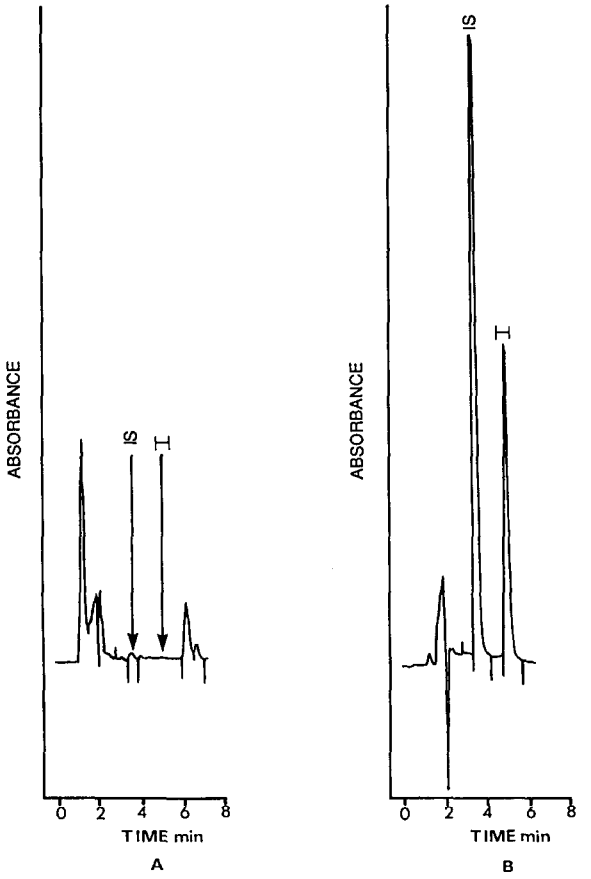


Fig. 5. Representative chromatograms of (A) blank plasma and (B) plasma spiked with internal standard (IS) and I (10 μg ml^{-1}).

both is greater than 90% at 0.5 μg ml^{-1}, and the C.V. at this concentration was 3.6%. Good recovery and precision are important for this assay as no internal standard was used. They are possible because loss through manual sample handling does not occur.

The recovery of II, II-A and other H^+/K^+ ATPase inhibitors of similar structure from spiked human serum have been shown to be dependent on pH [1,6]. Adjustment of serum pH on-line requires a buffer of sufficient molarity to overcome the natural buffering capacity of the sample. It has been reported that a 100 mmol l^{-1} buffer effectively adjusts serum pH when 200 μl are injected, whereas a 20 mmol l^{-1} buffer was not effective [6]. Higher buffer molarity is often used to allow a margin of safety and in their work Huber and Zech [1] used 300 mmol l^{-1} buffer when injecting 200 μl serum. However, unless very pure reagents are used, the limit of quantification of the assay can

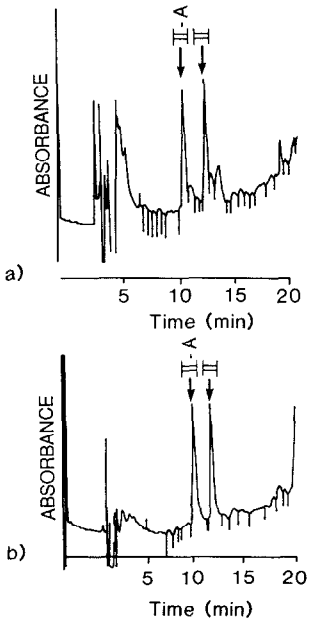


Fig. 6. Representative chromatograms of spiked plasma containing 1.0 μmol l^{-1} II and II-A injected directly into extraction column eluent made using 300 mmol l^{-1} buffer of different purity: (a) 300 mmol l^{-1} BDH AnalaR sodium acetate; (b) 300 mmol l^{-1} Merck Extra Pure sodium acetate.

be adversely affected by background originating from the buffer. This is of particular importance if only a small sample of 50 μl or less is available. A gradient chromatogram obtained using BDH sodium acetate AnalaR buffer (300 mmol l^{-1}) compared with Merck sodium acetate Extra Pure buffer (300 mmol l^{-1}) is shown in Fig. 6a and b, respectively.

When assaying small samples (50 μl) from a study involving sequential bleeds from rats the limit of detection could be improved by reducing the molarity of the buffer without loss of recovery. The recovery of II and II-A from a plasma sample (50 μl) using a C_2 extraction column and varying the molarity of the buffer was evaluated. Spiked plasma (50 μl) was injected directly into a flow of extraction eluent under identical conditions to those described for the HPLC of II and II-A above, but the molarity of the extraction eluent buffer varied. Stainless-steel tubing (20 cm $\times$ 0.009 in. I.D.) was fitted between the WISP and the 7010 valve to transfer the sample. The results showed that the recovery of II and II-A remained at greater than 90% until the buffer was less than 20 mmol l^{-1}. Below 20 mmol l^{-1} the pH of the sample was not lowered sufficiently to achieve good recovery of these compounds.

Sample preparation

The only sample preparation required for most biological fluids prior to introducing the sample to a Gilson–AASP or direct injection system is centrif-

ugation. However, dilution is performed off-line when assaying samples that have high drug concentrations, for example, following intravenous administration. It may also be necessary to simplify the matrix, for example by removing bile salts with ammonium sulphate [9] or proteins by precipitation with organic solvent [10]. Further manipulation such as the addition of internal standard, an organic modifier to free drug bound to proteins, or buffer to adjust the pH may be performed automatically [10]. However, when processing the sample automatically it is important not to allow any precipitation to occur, because this will block the extraction column.

Extraction column

The sorbents used for extraction columns have been derived from HPLC technology and there are many different phases available. The particle size is relatively large (25–40 μm) in comparison with that used for an analytical column. This is to allow large amounts of biological fluid to pass through without blocking the column. The average length of a column for on-line extraction is 10 mm and it contains 50–100 mg of sorbent. Using this large-pore-size material and a 10-mm column, the sample has to pass slowly through the column to achieve good recovery of the analyte. A flow-rate of approximately 1 ml min^{-1} is sufficient for most compounds.

Of the wide range of sorbents available, the reversed-phase C_2, C_8 and C_{18} are most popular [1]. However, for some applications a CN phase is preferred [11], and very selective extraction of diols has been achieved using a chemically bonded borate phase [12,13]. The authors suggest that if the analyte is lipophilic and has no potential to interact with selective phases a C_2 phase should be tried first. The advantage of this approach is that a C_2 phase will often retain the analyte but the background from endogenous compounds in plasma can be more easily removed than with the more strongly retaining C_8 and C_{18} phases. The analyte can also be eluted onto an analytical column more

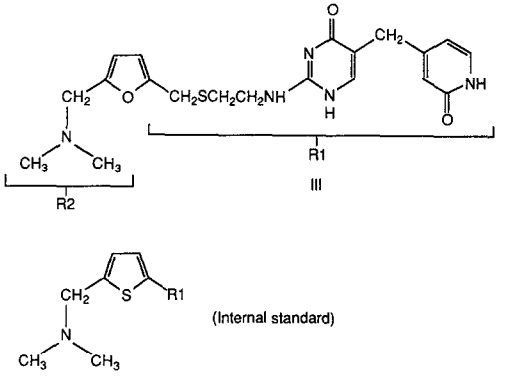


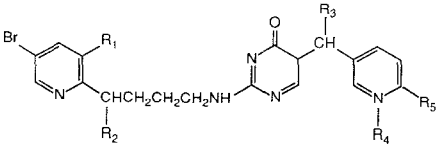
Fig. 7. Structures of III and internal standard.

easily from a C_2 phase than from higher phases which facilitates coupling of the extraction and analytical columns. This assumes that there is no interaction between the analyte and the residual silanol groups.

There is no definitive solution to the choice of extraction column, and the strong retaining power of C_{18} has been used to advantage in the assay of the H_2 receptor antagonist 5-(1,2-dihydro-2-oxo-pyrid-4-ylmethyl)-2-[2-[5-(dimethylaminomethyl)furanylmethylthio]ethylamino]pyrimidin-4(1*H*)-one-(III, Fig. 7) [9]. Although a base, III was extracted from plasma onto C_{18} sorbent at pH 4.5. It was completely retained, and the sample was washed with sodium carbonate (100 mmol l^{-1}), water and methanol–water (35:65, v/v). Methanol–water (80:20, v/v) was then used to remove III from the sorbent with greater than 90% recovery.

Analytical column

As described earlier, gradients are used for the assay of H^+/K^+ ATPase inhibitors and their metabolites of different polarity. Although gradients may reduce sample throughput compared with isocratic chromatography they are efficient for the assay of several analytes in a single chromatogram or for the resolution of compounds that cannot easily be resolved on an isocratic system. The authors have used a gradient for the assay of the H_1 receptor antagonist temelastine (IV, Fig. 8), five possible metabolites IV-A to IV-E and the internal standard, 2-[4-(3-methylpyrid-2-yl)butylaminol]-5-(4-chlorobenzyl)-4-



	R_1	R_2	R_3	R_4	R_5
IV	CH_3	H	H	-	CH_3
IV-A	CH_3	H	H	-	CH_2OH
IV-B	CH_2OH	H	H	-	CH_3
IV-C	CH_3	OH	H	-	CH_3
IV-D	CH_3	H	OH	-	CH_3
IV-E	CH_3	H	H	O	CH_3

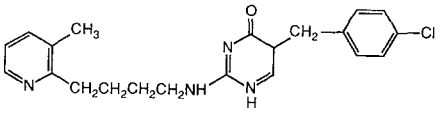


Fig. 8. Structures of IV, its internal standard and five possible metabolites (IV-A to IV-E).

pyrimidone [7]. For IV, plasma was extracted using a C_2 AASP cassette and eluted directly onto the analytical column using the gradient.

CONCLUSION

Two fully automated assays have been described to demonstrate the practical advantages of such systems for the handling of biological fluids. Routine systems such as the Gilson–AASP permit a series of mechanical manipulations to be performed, such as adjustment of pH and the addition of internal standard, prior to injection. Direct injection does not require specialised equipment and, provided sufficient consideration is given to mixing and avoidance of precipitation, is a most useful method. Routine systems such as these are both highly productive and leave the analyst free to develop the science rather than prepare samples manually for injection. Their use may seem complicated, but in practice time taken to learn them is only a few days. All the equipment is commercially available and is now developed to a point that it is reliable.

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