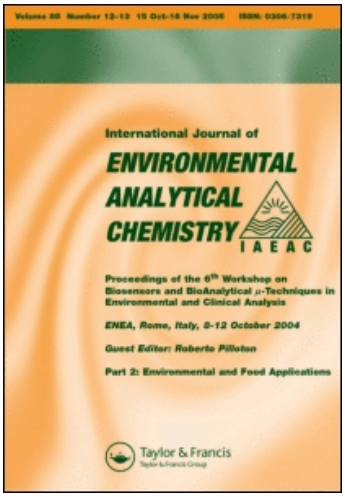

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Automated Extraction of Drugs from Biological Fluids†

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An automated continuous flow liquid–liquid extraction procedure is described for the separation of the H_2-antagonist loxtidine from plasma samples containing two metabolites which interfere in the radioimmunoassay of the drug.

The extraction of the bronchodilator salbutamol was studied using the DuPont Prep I automated liquid solid extraction apparatus, with a 12 cartridge capacity, and a vacuum extraction box designed in this laboratory to hold 30 Sep-pak C-18 (Waters Associates) cartridges. Twenty-four plasma samples per hour can be automatically processed with the Prep I. Although the vacuum box is not fully automated 45 plasma samples per hour can be processed. The Prep I can only be used with DuPont XAD, strong cation and anion exchange cartridges. Cartridges containing alumina, silica, florisil, cation and anion exchange resins and reverse phase packings can all be used with the vacuum extraction box. The latter costs only a fraction of the Prep I and therefore each analyst can have his own unit.

KEY WORDS: Automated extraction, drugs, biological fluids, metabolites, loxtidine, salbutamol.

INTRODUCTION

The pharmacokinetic studies necessary to evaluate the safety of a new drug in animals and its efficacy in man require the drug to be determined in a large number of samples e.g. blood, plasma, bile,

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saliva and urine. The drug is often present at the nanogram to microgram per ml concentration, whereas endogenous material e.g. plasma protein may be present at 70 mg/ml. Sometimes it is possible to analyse samples directly in biological fluids using e.g. h.p.l.c., and radioimmunoassay, but in most analyses the drug has to be separated from its metabolites and endogenous material. The main sample preparation procedures are based on:

a) Precipitation of protein followed by centrifugation and direct determination of the drug in the supernatant by spectrophotometric or chromatographic analysis.

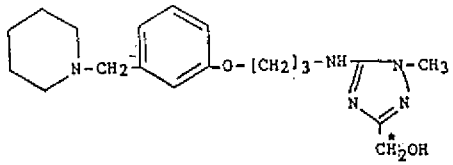
b) Extraction of the drug from the biological fluid or protein free solution into a water immiscible solvent at a pH dependent upon the pKa of the drug. The drug present in the organic solvent may then be determined by h.p.l.c., g.c., g.c.m.s., or r.i.a. In some instances it may be necessary to remove interfering material, e.g. metabolites, by back extraction of the drug into a suitable buffer. Liquid–liquid extraction procedures are time consuming and labour intensive. Also they use large quantities of glassware which has to be thoroughly cleaned before reuse.

c) Liquid solid extraction. Solid absorbents, e.g. charcoal, alumina, silica, XAD, cation and anion resins have been used for many years to separate drugs from biological fluids. The retained drug may then be extracted selectively from the absorbent by a suitable solvent and determined as described in Sections (a) and (b).

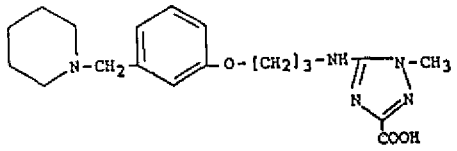
Automated systems are available for the injection of samples into gas and high performance liquid chromatographs, and computerised data collection and analysis systems are now fitted routinely to most chromatographic systems. These facilities have speeded up analyses, reduced the labour requirements and enabled chromatographic equipment to work unattended. What is now required is automated sample preparation.

The use of an automated liquid system in the analysis of the H_2-antagonist loxtidine Figure 1A is described. Also the DuPont Prep I[1] automated liquid solid extraction system is compared with a vacuum extraction box which can simultaneously process 30 samples for the determination of the bronchodilator salbutamol. Loxtidine, a tertiary base, has to be separated by manual solvent extraction from two acidic metabolites I and II which cross react with the antisera used to determine the drug by radioimmunoassay.[2] This is a slow

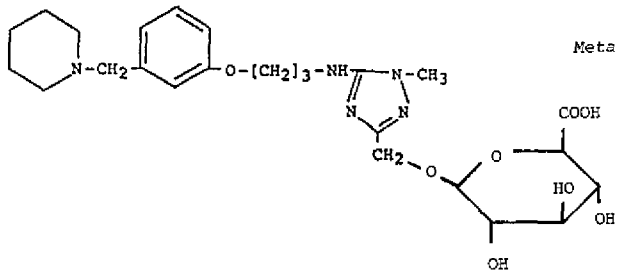
a)



Loxtidine

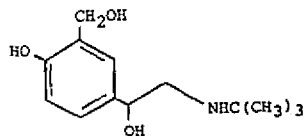


Metabolite I



Metabolite II

b)



Salbutamol

FIGURE 1 Structures of: (a) Loxtidine and Metabolites I and II and (b) salbutamol.

procedure and to speed up the analysis of plasma samples, an automated continuous flow extraction system was developed.

Organic solvents do not extract efficiently salbutamol, Figure 1B a very hydrophilic compound, from biological fluids. The extraction efficiency of salbutamol from biological fluids was improved using ion-pair reagents di-2-ethylhexyl phosphate, and tetraphenyl boron.[2]

This procedure is time consuming and alternative manual liquid–solid methods using XAD-2 and Sep-pak C18 (Waters Associates, Northwich, Cheshire, England) cartridges were developed. These have now been automated using the DuPont Prep I, which simultaneously processes twelve samples; and a vacuum extraction box, designed and manufactured in our laboratories which can process thirty samples simultaneously.

EXPERIMENTAL

An automated liquid–liquid system for the extraction of loxtidine from plasma

Loxtidine can be separated from its acidic metabolites with an efficiency of $86 \pm 0.7\%$ by extraction at pH 10 using dichloromethane. Initially this solvent was used for the automated liquid–liquid extraction system, but there were problems in the separation of the organic phase when the volume of plasma was increased to 300 μl. A series of solvents was studied and ethyl acetate saturated with ammonia gave the best separation and longest pump tube life. This reagent was prepared by shaking 1 volume of 3% v/v 0.880 ammonia in distilled water with 4 volumes ethylacetate. The phases were allowed to separate and the lower layer, Reagent A and the upper layer, Reagent B, used for the analyses.

Loxtidine was extracted from plasma samples (300 μl) with the continuous flow system using Autoanalyser system, Figure 2 (Technicon, Basingstoke, Hants, U.K.). A Technicon Sampler II fitted with a cam to aspirate 30 samples per hour, and Technicon pump I were used.

The aspiration time of the sample was controlled by a cam which allowed the probe to aspirate the samples for 80 seconds, then to transfer to the reservoir and aspirate water for 40 seconds. The time from aspiration of the sample to the arrival of its ethyl acetate extract at the collection tube was determined using plasma coloured with the lypophilic dye, sudan red. Studies with ^{3}H-labelled loxtidine showed that 4 minutes were required to collect the recoverable drug extracted from each plasma sample. Therefore a sample and water wash cup were placed alternately in the holes of the sampler plate. The water wash completely eliminated carry over of analyte between

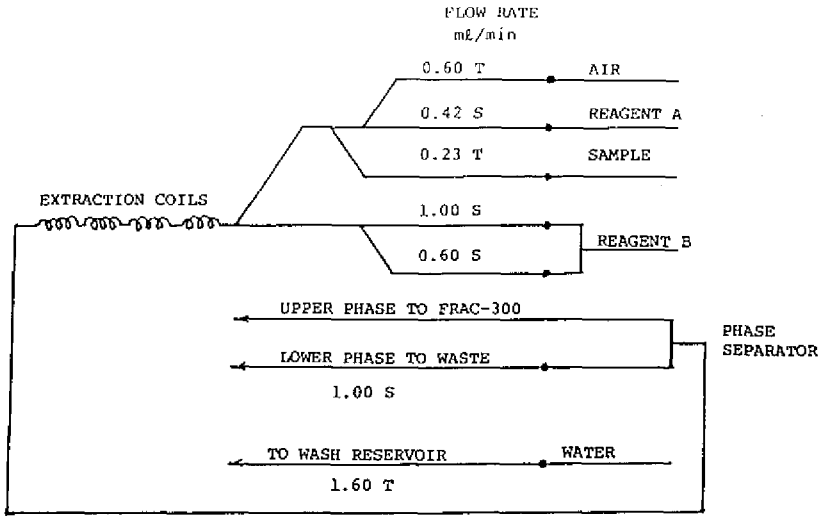


FIGURE 2 Manifold used for the automated liquid–liquid extraction of loxtidine. T=Tygon tube, S=silicone pump tube. Sampling rate 30/hr with a 2:1 wash ratio. Alternate sample cups contain water.

samples. A signal was obtained from a contact closure inside the sampler when it changed from aspirating from a sample cup to aspirating from the wash reservoir. This signal triggered an adjustable delay circuit in a timer unit which then activated the remote control terminal on the fraction collector, causing the collection head to move to the next tube at two minute intervals. The adjustable delay enabled the sampler and the fraction collector to be synchronised so that the drug was quantitatively collected in two consecutive 2.0 ml ethyl acetate fractions which were combined prior to evaporation.

The ethyl acetate extracts were evaporated to dryness using a heated vacuum centrifuge (Savant Speed-Vac concentrator, Model SVC 200, Savant instruments Inc., New York), with a capacity of 60 tubes, and the residues were dissolved in buffer and analysed by radioimmunoassay.[2]

Automated liquid solid separation systems for the separation of salbutamol from plasma

DuPont Prep I: Deuterated salbutamol, equivalent to a concentration of 50 ng/ml plasma, was added to each of the salbutamol calibration

standards used for the preparation of the standard line and to the plasma samples (2 ml) from subjects taking salbutamol. The Prep I is a microprocessor controlled centrifuge and evaporator and contains fifteen programmes to control the volume of wash and elution solvents, time of centrifugation, and evaporation. The machine is supplied with aluminium cups for evaporation of the solvent. These can be attacked by acidic or basic solvents used either for elution or by reagents used *in situ* for derivatisation of the residue prior to g.c., or g.c.m.s. The cups were replaced in our studies by screw capped glass tubes (15 mm i.d. × 35 mm). A plasma sample (2 ml) was pipetted into each Prep I XAD-2 resin cartridge. The lid of the instrument was closed and programme 15 selected. This centrifuged the plasma sample through the resin then washed the resin with 2 ml water. The microprocessor reversed the direction of rotation of the collection tube carrier, which brought an empty glass vial below the cartridge. Then 2 ml methanol was passed through the cartridge to elute the salbutamol.

In the earlier studies evaporation of the eluent was carried out automatically in the Prep I. To increase the sample throughput the vials containing the methanol eluates were removed from the Auto Prep and the solvent evaporated in a Savant evaporator.

Vacuum Extraction Box: A vacuum extraction box to take 30 cartridges has been designed and built in our laboratories, Figure 3. The box (300 mm long × 200 mm wide × 150 mm deep), capable of operating under vacuum without distortion, was made from stainless steel (3.2 mm thick) and was enclosed in a laminated case (A, Figure 3). The box has a vacuum inlet (B) and relief valves (C), a vacuum release safety valve (D) set to 380 mm Hg and a vacuum gauge (E, 0 to 760 mm Hg). The Perspex lid seals on a rubber gasket (F). The lid has thirty holes (5.6 mm i.d.) each with a rubber O-ring (BS 008) held in place by a metal strip (G). Prep I cartridges fit directly into the holes in the lid (H) but Sep-Pak cartridges (I) and Bond–Elut cartridges (J, Analytichem, Harbour City, California) require different adaptors, Figure 4, that extend into the collection tubes. A Perspex sheet (L) and a Perspex base (M) drilled to hold thirty sample collection tubes were suspended from the lid by four threaded posts (K, Figure 5). Different types of collection tubes, including liquid scintillation vials, could be used by changing the Perspex sheet and by raising the Perspex base by removing the spacers on the threaded

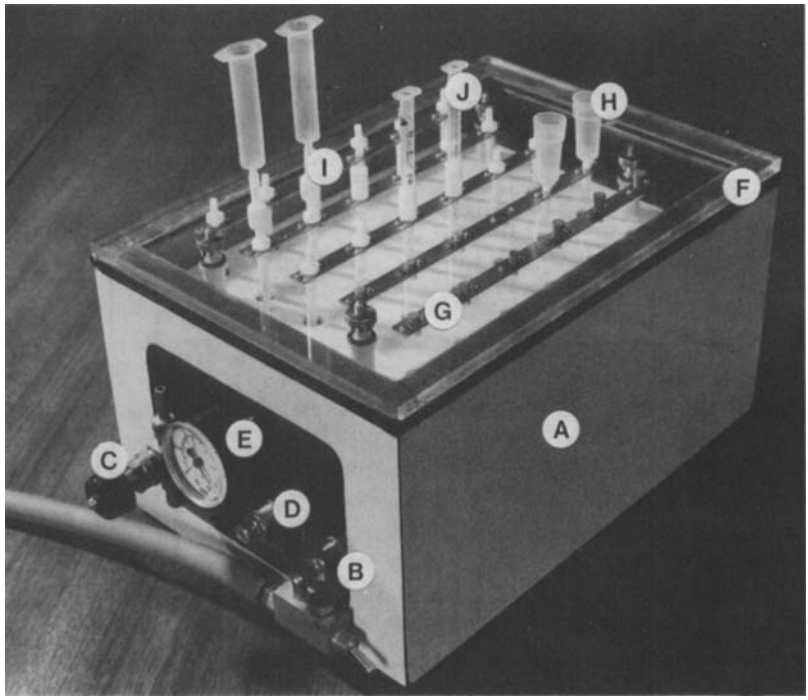


FIGURE 3 Vacuum extraction box. See text for details.

posts (K). For the conditioning of the cartridges the rack, Figure 5, was removed from the box and replaced by a metal tray (280 mm × 190 mm × 50 mm). A Sep-pak adaptor, Figure 4 (A) was fitted into each of the holes in the lid. A Sep-pak C-18 cartridge was fitted to each adaptor. A Luer tipped syringe was attached to each Sep-pak, Figure 3. First methanol (5 ml) was added to each syringe, and a vacuum was applied to draw the solvent through the cartridge. Then water (5 ml) was aspirated through the column. Each salbutamol standard in plasma and the plasma sample for analysis (2 ml) were pipetted into a syringe barrel and drawn through the cartridges which were then washed with water and acetonitrile. The vacuum was released and the tray containing the eluents removed. Collecting tubes were fitted into the carrier, Figure 5 which was attached to the Perspex lid. The lid was replaced on the box, methanol 2 ml was placed into the syringe barrel attached to each

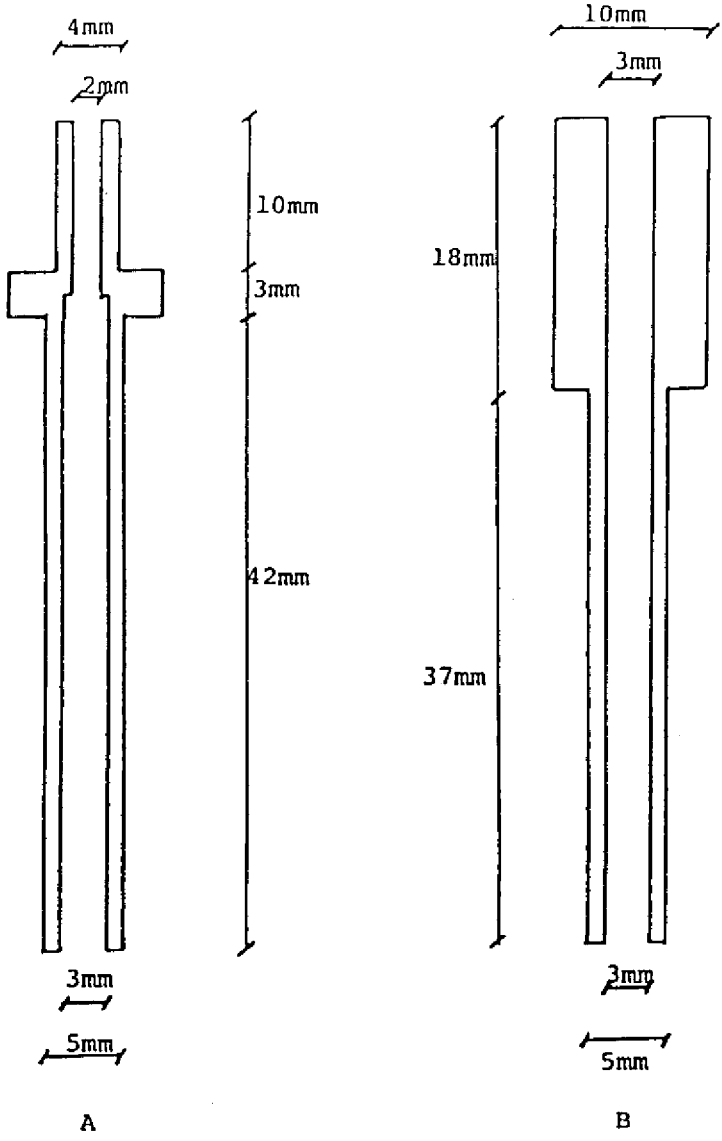


FIGURE 4 Diagram of the adaptors used for (a) Sep-Pak cartridges and (b) Bond-Elut cartridges.

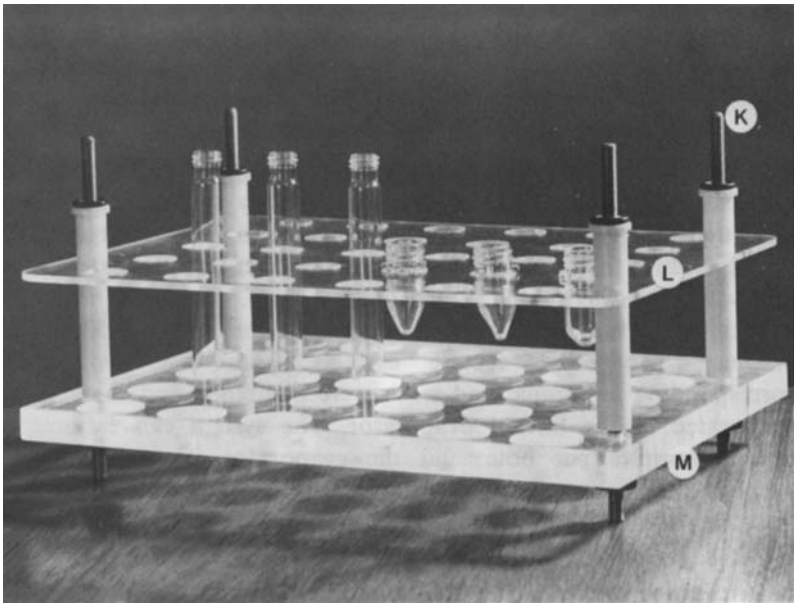


FIGURE 5 Collecting tube rack removed from the vacuum box. See text for details.

cartridge and a vacuum applied. The flow rate through the cartridge for the washed sample and solvents was adjusted to below 3 ml per minute. The vacuum was released and the tubes containing the methanol eluent were transferred to the Savant evaporator for removal of the solvent.

The salbutamol in the residue obtained from both extraction procedures was determined by g.c.m.s.[3]

RESULTS AND DISCUSSION

Liquid–liquid extraction—separation of loxtidine from plasma

In the continuous flow extraction system the ethyl acetate was pumped at 1.6 ml per minute, but to prevent build up of protein in the separator and eliminate carry over of the aqueous phase, only 1 ml per minute, i.e. 62.5% of the ethyl acetate was collected. There can be carry over of analyte from one sample to another and to

prevent this, a cup containing water was placed between each sample. The time for the sample to pass through the manifold was established using the sudan red method, the carry over between samples, synchronisation and efficiency of extraction was determined using a 7% bovine serum albumin solution containing 100 ng ^{3}H-loxtidine/ml, i.e. 4×10^3 dpm. Radiochemical analysis of the collected fractions showed that 90% of the radioactivity was present in the collection tube corresponding to the sample and 9% in the next tube corresponding to the wash. The overall recovery of ^{3}H-loxtidine in the two tubes was $58 \pm 1.5\%$ (theoretical 62.5). Loxtidine has been quantitatively recovered over the concentration range 5–500 ng/ml plasma.[2] The ethyl acetate extracts from the two tubes corresponding to sample and wash were combined and the solvent evaporated in the Savant evaporator. The system can extract 15 plasma samples per hour and the evaporator takes 1 hour to evaporate the solvent from 60 samples. The residue in each tube was dissolved in buffer and determined by radioimmunoassay.

Liquid solid extraction—of salbutamol from plasma Prep I

It was possible to process 24 samples per hour using the Prep I. The efficiency of extraction of salbutamol was $93 \pm 2\%$ ($n = 6$) at a concentration of 50 ng salbutamol/ml. Tris-deuterated salbutamol was added at a concentration of 50 ng salbutamol/ml to plasma as an internal standard for the determination of salbutamol by g.c.m.s. This acted also as a carrier for salbutamol and using this extraction procedure salbutamol could be quantitatively recovered from plasma at from 0.25–50 ng/ml plasma. The throughput was increased to 48 per hour by carrying out the evaporation in the Savant evaporator instead of the Prep I. When these studies were carried out only DuPont XAD-2 cartridges were available for use in the Prep I and this limited its use. More recently strong anion and cation resins have been marketed by DuPont and the use of these is being investigated.

Vacuum extraction box

The vacuum extraction box processed 30 samples simultaneously with a throughput of 45 per hour and similar efficiency to the Prep I. The advantages of the vacuum box over the Prep I were:

a) The vacuum box costs only a fraction of that of the Prep I, therefore, each analyst can have his own box.

b) Commercially available cartridges, from different manufacturers, containing alumina, silica, florsil, ion exchange resins and reverse phase packings can be used with the vacuum box.

c) When developing new methods of analysis, different cartridges and solvents can be evaluated in the same batch of analyses.

The disadvantage with the vacuum box is that the wash and extraction solvents have to be pipetted manually on to the cartridges. The use of commercially available programmable diluters to dispense the solvents would make the method more automated.

CONCLUSION

The automated liquid–liquid extraction system was as efficient as the manual method for extracting loxtidine from plasma. Unattended it can process 15 plasma samples per hour. The Prep I and vacuum extraction box were equally effective at extracting salbutamol from plasma when liquid solid systems were used. The Prep I can automatically process 24 samples per hour. This could be increased to 45 samples per hour if the methanol eluents were removed and concentrated in an external evaporator. Forty-five samples per hour can also be processed using the vacuum box. The Prep I is restricted to the use of DuPont XAD-2 and strong cation and anion cartridges, whereas the vacuum box can be used with all the commercially available cartridges which cover a wide range of adsorbants.

Acknowledgement

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