Journal of Chromatography, 317 (1984) 475–481
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMSYMP. 519

HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC DETERMINATION OF OXMETIDINE IN HUMAN PLASMA: COMPARISON OF LIQUID–LIQUID AND LIQUID–SOLID EXTRACTION TECHNIQUES

R. D. McDOWALL*, G. S. MURKITT and J. A. WALFORD
Department of Drug Analysis, Smith Kline & French Research Ltd., The Frythe, Welwyn, Herts AL6 9AR (U.K.)

SUMMARY

A preliminary liquid–solid sample preparation scheme (LSE) for the HPLC determination of oxmetidine in human plasma is compared with the existing liquid–liquid extraction. The LSE method shows great practical advantages, such as ease of preparation, saving of time, and smaller sample volumes, but needs to be investigated further with respect to robustness and the removal of an endogenous compound that interfered with the quantitation of oxmetidine at low concentrations.

INTRODUCTION

The determination of drugs in biological fluids by modern analytical techniques usually requires a purification and enrichment step which removes endogenous material with the potential of interfering with the assay, while it concentrates the analytes so that they can be detected and quantified. Liquid–liquid extraction (LLE) is a method commonly used by analysts. The advantage of this technique is one of selectivity, but the main disadvantages are emulsion formation, which gives lower recoveries of analytes, and the time taken to perform the multiple liquid transfers required. Thus, the histamine H_2 receptor antagonist cimetidine has been extracted from plasma by a variety of organic solvents, including methylene chloride[1–3], ethyl acetate[4,5] and octanol[6,7], which has also been used to extract ranitidine[8], SK&F 93479[9], and oxmetidine[10,11].

The extension of the use of silica, with a bonded organic phase, from high-performance liquid chromatography (HPLC) to sample preparation now offers a viable alternative to LLE[12]. This approach improves the speed and ease of sample preparation while it retains the efficiency in purification and concentration.

Cimetidine is the only histamine H_2 receptor antagonist for which a liquid–solid extraction (LSE) method exists[13], it involves the use of mini-columns, packed with C_{18} bonded silica (Sep-Pak, Waters Assoc., Milford, MA, U.S.A.) for preparing plasma and urine samples for HPLC analysis. The method is quicker than multiple solvent extraction, as efficient, and does not require expensive high-purity organic solvents.

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We present here a preliminary LSE scheme, with the aid of C_{18} cartridges as a useful means of processing human plasma samples for the analysis of oxmetidine by HPLC; the new sample preparation process is compared to the more conventional LLE[11].

EXPERIMENTAL

Materials

All chemicals used in this study were of analytical grade, with the following exceptions: 1-octanol was puriss (Koch-Light, Colnbrook, U.K.); methanol, water and acetonitrile were HPLC grade (Rathburn, Walkerburn, U.K.); sodium 1-pentanesulphonate was reagent grade (Kodak, Rochester, NY, U.S.A.). The solutions of 1 *M* carbonate buffer (pH 9.0 and 10.0) and 0.1 *M* acetate buffer (pH 5.0), and the collection of control human plasma were described previously[11]. Subtilisin Carlsberg (protease type V111) from *Bacillus subtilis* (ref. P5380) was supplied by Sigma (St. Louis, MO, U.S.A.).

The stock solution of oxmetidine for the plasma assays was prepared by weighing 5.91 mg of the dihydrochloride salt (equivalent to 5 mg of base), and dissolving it in approximately 0.5 ml water before making up to 100 ml volume with methanol. Solutions of the internal standard {SK&F 93586, 2-[2-(5-methyl-4-imidazolyl-methylthio)-ethylamino]-1-methyl-5-(3,4-methylenedioxybenzyl)-6-pyrimidone dihydrochloride} were similarly prepared by weighing 11.77 mg (equivalent to 10 mg base) and dissolving it in the appropriate volumes of water and methanol described above.

The polypropylene centrifuge tubes (12 ml) and stoppers used for LLE were obtained from Henleys Medical Supplies, London, U.K. (type 300PP and 301PT, respectively).

The disposable solid-phase columns, 3-ml reservoirs, and adaptors for LSE were obtained from Analytichem International, Harbor City, CA, U.S.A. (100 mg C_{18} Bond Elut, Part No. 607101, reservoirs Part No. 6004). These were affixed to a vacuum manifold (Vac-Elut, Analytichem International) to accelerate the passage of solvent through the sorbent bed.

Extraction procedures for plasma

The LLE of oxmetidine from human plasma is as previously described[11]; it requires 2 ml of plasma.

The scheme for extraction of plasma by LSE is as follows: the cartridge is activated by passing 1 ml methanol followed by 1 ml water through the sorbent bed. One ml of plasma containing 2.5 μg SK&F 93586 as internal standard and 0.5 ml carbonate buffer (pH 10.0, 1 *M*) is then passed through the cartridge by the application of vacuum. The column is washed with 2 ml water to remove any traces of plasma and to elute any water-soluble compounds. The vacuum is increased to 25 inch Hg and air is drawn through the cartridge to dry the sorbent bed for 2 min by removing the majority of aqueous phase. Oxmetidine and the internal standard are eluted into small polypropylene recovery tubes by the application of methanol (250 μl) to each column, the solvent is transferred to an auto-sampler vial containing a limited volume insert and stored at -20°C pending HPLC analysis. Throughout the procedure the vacuum applied to the Vac-Elut manifold for drawing fluid through the cartridge was maintained between 5 and 8 inch Hg.

Chromatographic operating conditions

The chromatograph consisted of a Model 6000A pump (Waters Assoc.). The sample extract was introduced into the system via an automatic injector (Model WISP 710B, Waters Assoc.). Sample extracts were held in spring-loaded microinserts (Type 3-CV, Chromacol, London, U.K.) 4-ml vials with self-sealing septa (Cat. Nos. 73018 and 73010, respectively, Waters Assoc.). The analytes were separated on a stainless-steel column 150 × 4.6 mm I.D. packed with 5 μm Ultrasphere ODS (Beckman, CA, U.S.A.) and maintained at 40°C. The column effluent was monitored by a Model 773 (Kratos Instruments, Manchester, U.K.) variable-wavelength detector set at 226 nm and 0.01 absorbance units full scale. The signal from the detector was fed into a Model 301 integrator (Laboratory Data Control, Stone, U.K.).

The solvent system was a mixture of water–methanol–acetonitrile (45:44:11, v/v) containing 0.095 *M* pentanesulphonic acid and prepared as follows: 17.33 g sodium pentanesulphonate was dissolved in 450 ml distilled water, and the pH of the solution was adjusted to 3.0 with 10 *M* sulphuric acid; 440 ml methanol and 110 ml acetonitrile were added and dissolved air was removed by the application of reduced pressure. The column was equilibrated by passing solvent through it for approximately 1 h before commencing the analysis. Upon completion of analysis it is recommended that the column be flushed with filtered methanol for 1–2 h.

At a flow-rate of 1.0 ml min^{-1} the approximate retention times of oxmetidine and SK&F 93586 (the internal standard) were 5 and 6 min, respectively.

Quantification

The area under each peak was determined by an integrator connected to the UV detector, and the ratios of the peak areas assigned to oxmetidine and the internal standard were calculated. The concentration of oxmetidine was then calculated from calibration curves obtained with standards containing known amounts of oxmetidine.

RESULTS AND DISCUSSION

Recovery

Recovery of spiked oxmetidine from 2 ml plasma by the LLE technique averaged 60% (the theoretical recovery after allowance for the volumes taken was 75%)[10].

Recovery from aqueous solutions by LSE averaged 90% but fell to 50% in plasma. The major factor affecting recovery was initial binding to the column: in plasma 40% of the added drug was not retained. This breakthrough could have occurred because the plasma contained compounds that preferentially bind to the ODS-silica thereby excluding the drug or because oxmetidine was protein-bound to an extent which affected the capability of the drug to bind to the sorbent.

To investigate the latter aspect plasma was incubated at 55°C for 1 h with subtilisin Carlsberg to hydrolyse protein (0.5 mg enzyme added to 0.5 ml pH 10 carbonate buffer), this increased the recovery of drug by 20% (measured by ^{14}C-oxmetidine) but released endogenous material that obliterated the peaks of interest on the chromatogram. By increasing the amount of C_{18} used in the Bond Elut column to 500 mg we also showed that the breakthrough was partly due an insufficient amount of sorbent. However, the increased volume of methanol (500 μl) required to

elute the analytes meant that it needed to be evaporated; this produced interfering peaks on the chromatogram which precluded quantitation of oxmetidine.

Selectivity

Samples of up to 10 μl of the ethanol extracts from the LLE method were injected into the chromatograph to obtain separation of the peaks of interest; the injection of more than 20 μl ethanol often resulted in loss of resolution. No unwanted peaks with relevant retention times were observed in plasma extracts. Typical chromatograms are shown in Fig. 1. The methanolic extracts from the LSE are more compatible with the mobile phase than ethanol. Injections of up to 100 μl of standard solutions can be made without the loss of resolution or peak shape. Utilizing the present solvent system, we are again limited to a 10-μl injection, because an endogenous peak affected the accurate measurement of oxmetidine. Typical chromatograms are presented in Fig. 2.

Selective removal of the endogenous peak by modifying the extraction procedure has up to now proved unsuccessful because the retention of the interfering substance has proved to be very similar to that of the analytes under a variety of conditions. We tried chromatographic mode sequencing (CMS) where the analytes were eluted from the C_{18} column with methylene chloride, then sorbed on a 100-mg diol column, dried, and finally eluted with 250 μl mobile phase. Because this gave an overall recovery of 5% from plasma, required additional time and an additional adsorption column, no further work was undertaken. We are however, analysing

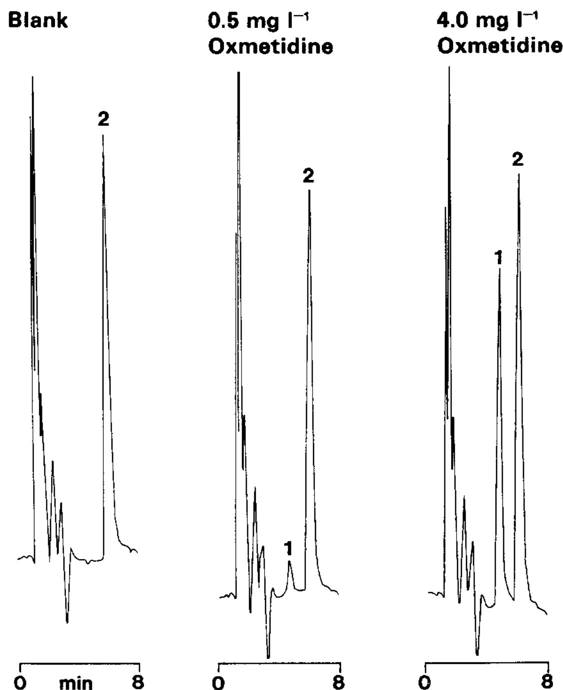

Fig. 1. HPLC chromatograms of LLE extracts. Peaks: 1 = oxmetidine; 2 = SK&F 93586 (internal standard).

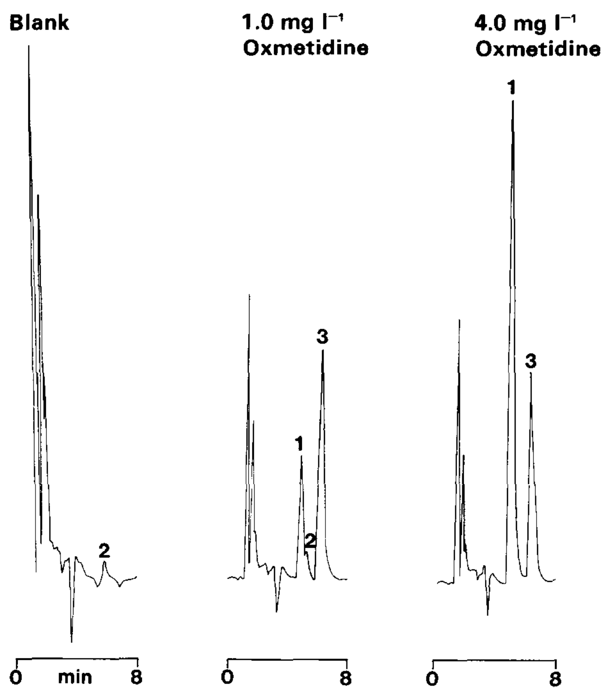

Fig. 2. HPLC chromatograms of LSE extracts. Peaks: 1 = oxmetidine; 2 = endogenous peak; 3 = SK&F 93586 (internal standard).

TABLE I

PRECISION AND ACCURACY OF THE LLE AND LSE PROCEDURES

Bias calculated as $\frac{\text{(mean calculated concentration} - \text{actual concentration)}}{\text{actual concentration}} \times 100\%$

Oxmetidine concentration ($mg\ l^{-1}$)	*Mean calculated concentration ± S.D. ($mg\ l^{-1}$)*	*Coefficient of variation (%) (n)*	*Bias (%)*
LLE			
0.25	0.29 ± 0.04	13.8 (10)	17.6
0.50	0.55 ± 0.04	7.3 (10)	10.0
2.50	2.53 ± 0.07	2.8 (10)	−1.2
5.00	5.02 ± 0.06	1.2 (10)	0.4
LSE			
0.20	0.29 ± 0.005	1.7 (4)	+45.0
1.00	0.85 ± 0.08	9.8 (6)	−15.0
2.00	1.84 ± 0.16	8.7 (6)	−8.0
3.00	2.90 ± 0.14	4.8 (6)	−3.3
4.00	3.95 ± 0.10	2.5 (6)	−1.3
5.00	5.05 ± 0.34	6.7 (6)	1.0

extracts derived from a LSE clean-up procedure by HPLC optimised for samples from LLE. As a consequence this system should be optimised for LSE sample extracts.

Precision and accuracy

The precision and accuracy of the two methods of sample preparation are presented in Table I. The precision of the LSE method, expressed as coefficient of variation (CV, %), was between 2.5 and 9.8% over the concentration range 1.00–5.00 mg l^{-1}. This variation was slightly larger than that for the LLE method. Accuracy as measured by bias was acceptable between 1.0 and 5.0 mg l^{-1} for the LSE method. Below 1.00 mg l^{-1} the unacceptably large bias (+45%) was the result of the presence of the endogenous peak seen in Fig. 2.

Determination of unknown samples

Twenty tubes, containing human plasma were spiked with various concentrations of oxmetidine and then extracted by the LSE and LLE methods outlined above. The results are shown in Fig. 3 below.

The calculated results for the first ten samples show good agreement with the actual concentrations of oxmetidine; these samples were all processed simultaneously on the Vac-Elut manifold. The second set of 10 samples, also processed together, show a different picture; the calculated results are usually lower than the added concentration. The reason for this is not known. The columns were all from the same batch, and the vacuum conditions were kept within the ranges stated in the method. The flow-rate of fluid through the column will affect adsorption, and it may be prudent to narrow the range of pressures needed to adsorb and elute the analytes from the column to a single value.

Practical considerations of the two extraction methods

There is no doubt that the LSE method is far easier, simpler and quicker than LLE. Ten samples are processed simultaneously by LSE, each wash or elution step being achieved simply by the addition of the appropriate solvent to the head of the column. In comparison, many laborious liquid transfer steps are necessary in the

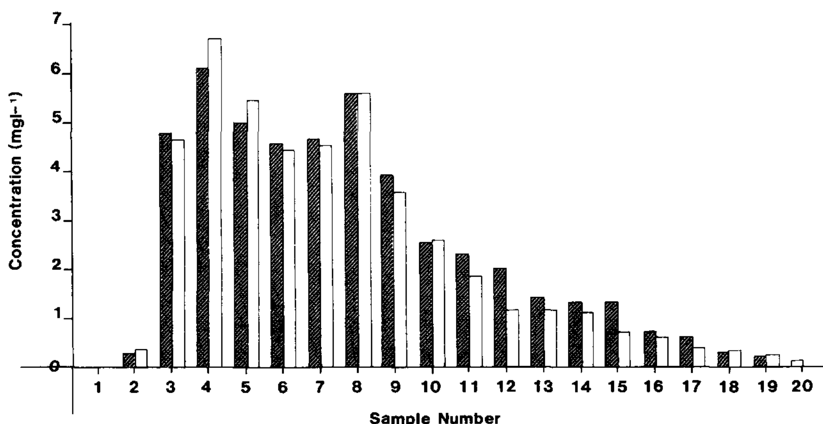

Fig. 3. Comparison of added and calculated oxmetidine concentrations obtained after LSE and LLE analysis. (■) Added concentration; (□) calculated concentration.

traditional LLE technique. The new method also avoids the use of toxic or hazardous organic solvents and the risk of emulsion formation. The time taken to process 60 samples by LSE was 1.5 h, compared with 5 hours by LLE.

CONCLUSIONS

LSE offers distinct practical advantages over the conventional method of sample preparation with respect to the time, effort, and sample volume required. However, investigations are needed to examine the robustness of LSE, particularly with respect to the variation seen between different batches of samples analysed on the Vac-Elut columns. Investigation into the removal of the endogenous interfering peak either by further purification or by alteration of the HPLC conditions is essential before this LSE method can be used in pharmacokinetic studies.

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