Research Article

Microemulsion High Performance Liquid Chromatography (MELC) for Determination of Terbutaline in Urine Samples

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ABSTRACT

An isocratic oil-in-water microemulsion High Performance Liquid Chromatography (MELC) was developed and validated for the determination of terbutaline in urine samples. A solid phase extraction (SPE) method which used Oasis HLB cartridges was optimised to isolate terbutaline from a urine matrix followed by HPLC with fluorescence detection. The urinary assay was performed in accordance with FDA and ICH regulations for the validation of bioanalytical samples. The method uses the isocratic oil-in-water micro emulsion to separate the terbutaline from the endogenous urine components. The chromatographic separation was carried out on C18-Spherisorb (250mm×4.6mm) analytical column maintained at 30 °C. The mobile phase was 94.4% aqueous orthophosphate buffer (adjusted to pH 3 with orthophosphoric acid), 0.5% ethyl acetate, 1.5% Brij35, 2.5% 1-butanol and 1.1% Octanesulfonic acid (OSA), all w/w. The terbutaline peak was detected by fluorescence detection, using excitation and emission wavelengths of 267 and 313 nm, respectively. The linearity of response was demonstrated at six different concentrations of terbutaline which were extracted from spiked urine, ranging from 60 to 1000ng/ml. The terbutaline was extracted from urine by a solid phase extraction clean-up procedure on Oasis HLB cartridges, and the relative recovery was >87.64% (n = 5). The limit of detection (LOD) and limit of quantitation (LOQ) in urine were 20.21 and 61.24ng/ml, respectively. The intra-day and inter-day precisions (in term of % coefficient of variation) were <3.56% and <2.87%, respectively. In the method development the influence of the composition of the microemulsion system was also studied and the method was found to be robust with respect to changes of the microemulsion components.

Keywords: Assay; Validation; Terbutaline; Microemulsion; HPLC;MELC; Urine.

INTRODUCTION

Determination of beta-agonists compounds in human urine samples by isocratic reversed-phase HPLC can be very difficult and challenging because of the presence of proteins, acidic and basic compounds, and other organic compounds that have a similar chemistry to the analyte of interest. Also, the presence of silanol groups on the surface of the column can affect the peak shape and hence reduce resolution between the analyte and urine matrix components.

Microemulsion High Performance Liquid Chromatography (MELC) has previously been used for the separation of mixtures of test solutes and also for the separation of drugs and their related impurities in pharmaceutical formulations[1-6]. However, very few studies have used MELC to separate drugs in biological fluids[7]. Furthermore, very few of these studies have validated HPLC methods for separation in biological fluids.

Polettini[12] have reported a coupled-column liquid chromatography for the analysis of β-agonists (terbutaline) in urine by a direct sample injection method. However, in the method the direct injection of urine, a large injection volume, a very high flow-rate and length of the columns used cause a very high operating backpressure and short column life.

Several other methods were reported for the analysis of terbutaline and for the determination of terbutaline enantiomers in urine samples using conventional HPLC[8-11]. However, the main drawback of these methods is the lengthy analysis run-time and also the organic solvent waste of the mobile phases which increases the burden on the environment, as well as being costly to dispose of[13,14]. The objective of the current work was to apply MELC for the analysis of terbutaline in urine samples and explore the potential of MELC for rapid separation of terbutaline from urine matrix components.

MATERIALS AND METHODS

Solid Phase Extraction (SPE)

The method reported by Mazhara[8] was adapted for the extraction of salbutamol from urine samples. A pretreated sample was prepared by adding 1ml of an aqueous solution of 600µg/l (w/v) bamethane to 1ml of urine as an internal standard. The samples were buffered with 2mL of 30m MKH2PO4 (pH 7.0) and vortex mixed. The pH of the pre-treated samples (4 mL) was checked. Each Oasis HLB cartridge was conditioned with 2ml methanol followed by 2ml of 45mM potassium dihydrogen phosphate (pH 7). The pretreated sample was then applied to the cartridges followed by the addition of 2ml of 15mM potassium dihydrogen phosphate (pH 7) continued by full vacuum drying for 2 min. The cartridge was then washed with 1ml of MeOH:H_2O (5:95 v/v) followed by full vacuum drying for 1 min, then 1ml of ACN:H_2O (02:98v/v) continued by full vacuum drying for 1 min, then 1ml of THF:H_2O (0.25:99.75v/v) followed by full

vacuum drying for 2 min. The analyte was then eluted from the cartridge into a sample tube using 3ml of $CH_3COOH:H_2O$ (02:98v/v) at low vacuum (less than 3 bar) for 2-3 min.

After evaporation to dryness using a dry nitrogen stream at 120°C for 30 min, the residue was reconstituted in 1ml mobile phase and 100µml was injected into the HPLC system.

Preparation of mobile phase

The standard micro emulsion system was prepared by weighing 1.5g Brij35, 0.5g ethyl acetate, 2.5g butanol, 1.1g octane sulfonic acid (OSA) and 94.4g of potassium dihydrogen phosphate buffer 20mM to a 100ml flask.

This mixture was sonicated for 15 minutes to complete dissolution. The solution was an optically transparent micro emulsion, which was stable for several months.

Terbutaline urine solutions

A stock solution containing 50mg/L of terbutaline was prepared using bamethane (Sigma, UK) as internal standard. The internal standard was prepared beforehand at a concentration of 400µg/L in blank urine.

The urine blank was collected from six different human sources, including male and female subjects. Calibration standards of terbutaline in the concentration range 60–1000ng/ml were prepared using internal standard urine solutions.

Method development

The concentration of the surfactant

A blank urine sample from human subjects was extracted and afterwards was spiked with terbutaline and bamethane. The components of the micro emulsion were obtained by experimentation and the Brij35 was found to have a small effect on retention time and on the separation resolution (Table 1). The optimum micro emulsion consisted of: Brij35 1-2%, ethyl acetate 0.5%, butanol 0.75%, phosphate 95.5% pH3.

Table 1: Effect of Brij35 concentration on retention time of terbutaline and bamethane.

Brij35 (% w/w)	Terbutaline	Bamethane
0.5	4.2	6
1	4.3	5.1
1.5	4.3	5.1
2	3.9	4.9

The concentration of the co-surfactant

Co-surfactants are usually used to assist the formation of the micro emulsion and this is considered an important factor in stabilizing the micro emulsion system[5,6]. The effect of butanol (co-surfactant) on the retention behaviour of spiked urine samples with terbutaline and bamethane was studied. It was found that retention time of both terbutaline and bamethane decreased on increasing the concentration of butanol (Table 2).

Table 2: Effect of co-surfactant concentration on retention time profile of terbutaline and bamethane.

Butanol (% w/w)	Terbutaline	Bamethane
0.5	6.7	8.2
1.5	5.2	7.1
2.5	4.3	5.1
3.5	4.3	5.1

Effect of Oil

The effect of oil (ethyl acetate) concentrations on the separation of terbutaline spiked urine samples containing internal standard was examined. It was found that increasing the oil concentration had no marked effect on the retention time of either terbutaline or bamethane (Table 3). The microemulsion mobile phase consisted of: SDS 1.6%, Brij35 1.6%, octane 1.25%, butanol 8%, phosphate pH 3 87.5%.

Table 3: Effect of oil on the solute retention time of terbutaline and bamethane.

Ethyl acetate (% w/w)	Terbutaline	Bamethane
0.5	4.3	5.1
0.75	4.3	5.0
1	4.1	4.9

Effect of ion pair

The effect of the addition of different types of ion pair agent on separation selectivity was examined using heptane sulfonic acid (HSA) and octane sulfonic acid (OSA). Table 4 shows the concentration of the examined ion pairs solutions that gave the best separation of both terbutaline and bamethane from the urine matrix. It was found that OSA has a more significant effect both on retention time and separation resolution as compared to HSA. The optimum micro emulsion was found to be 1.5% Brij35, 0.5% ethyl acetate, 2.5% butanol, 1.1% octane sulfonic acid in 0.02Mphosphate buffer pH 3. This MELC mobile phase gave a separation of the terbutaline and the internal standard (bamethane) from the urine matrix in a reasonable analysis time (less than 15 min).

Table 4: Effect of addition of different types of ion pair on solute retention.

Factors	Terbutaline	Bamethane
HSA (ion pair) 4 %	7.0	9.0
OSA (ion pair) 1.1%	9.8	13.6

Method validation

The method was validated by determining levels of terbutaline in the human urine samples. For this ethical approval was obtained from the University of Bradford.

The validation procedure was based on ICH guidelines for bioanalytical method validation[15].

Specificity

Specificity was tested by quantifying terbutaline in the presence of other components in the urine matrix. Blank urine samples were collected from six (3 females, 3 males) volunteers were individually spiked with terbutaline. Both the blank and spiked urine samples were extracted by HLB-SPE (see section 2.1) from the urine matrix and then assayed by HPLC.

The MELC mobile phase was also compared with other conventional mobile phases, i.e.methanol:OSA:phoshate buffer at pH3 [20:1.1:78.9 v/v] and a micellar mobile phase which consists of [1.5%brij35: 2.5% butanol:1.1% OSA:94.9% v/v buffer pH3](Figure 3). The specificity of the method (MELC) showed that there were no interferences from components of endogenous material. Peaks of terbutaline and internal standard were well resolved and were separated from the urine components (Figure 1 and Figure 2). On the other hand, the conventional mobile phase and micellar failed to separate terbutaline and bamethane from urine matrix components.

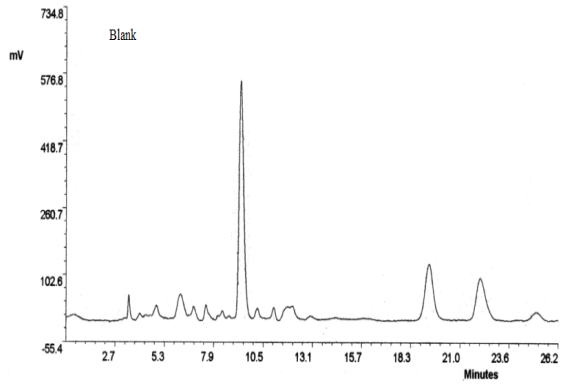

Figure 1: Chromatogram obtained from blank urine after the extraction. Micro emulsion consists of: Brij35 1.5%, ethyl acetate 0.5%, butanol 2.5%, OSA 1.1%, phosphate pH3 94.4%, at 25˚C, 1.0 ml/min flow rate and C18 column ODS1.

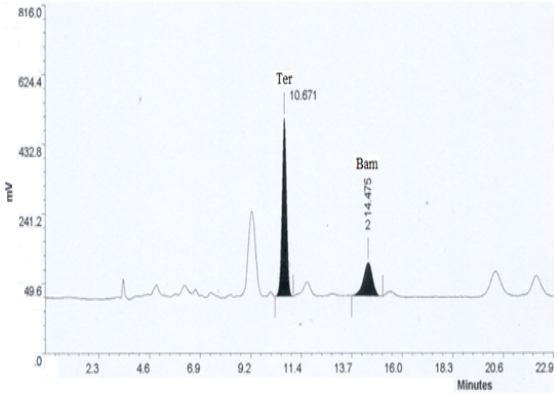

Figure 2: Chromatogram obtained from terbutaline spiked urine (400ng/ml) after the extraction. Micro emulsion consists of: Brij35 1.5%, ethyl acetate 0.5%, butanol 2.5%, OSA 1.1%, phosphate pH3 94.4%, at 25˚C, 1.0 ml/min flow rate and C18 column ODS1.

Linearity

Different concentrations of terbutaline ranging from 60 to 1000ng/ml including the lower limit of quantification (LOQ) and covering the predicted range were prepared in the urine matrix. Terbutaline was extracted as above with HLB-SPE. Blank samples were also analyzed along with the calibration standards. The extracted terbutaline samples including internal standard were injected in duplicate. The calibration curve showed a linear response and gave a regression coefficient (r^2) of 0.9981 (y = 0.0088x + 0.2358).

Sensitivity

The limit of detection (LOD) and limit of quantitation (LOQ) of the method for terbutaline spiked urine samples were calculated from the mean of the intercept of five urine calibration curves. The LOD and LOQ were 20.21 and 61.24ng/ml respectively. Terbutaline in urine at a concentration of 60ng/ml was extracted then injected on five separate days to determine the coefficient of variation of the terbutaline peak.

Precision

The precision was examined by five determinations at each of the known concentrations corresponding to low (200ng/ml), medium (600ng/ml) and high (1000ng/ml) levels in the calibration range. This study was repeated for five days to determine the inter-day variation. Inter-day assay CVs ranged from 2.87% to 4.28% at concentrations ranging from 200 to 1000ng/ml (Table 5).

Recovery

The recovery was calculated by comparing the peak area of the extracted terbutaline urine standard at three concentrations levels: low (200ng/ml), medium (600ng/ml) and high (1000ng/ml), with the peak area of the terbutaline standards externally spiked with the extracted blank urine matrix. The measurements were performed using five determinations of each concentration. The method showed excellent and consistent recoveries, and the mean recoveries for the studied concentrations ranged from 87.64 to 94.02% (Table 6).

Accuracy

The accuracy of the method was assessed by replicate analysis (n=5) of the extracted terbutaline urine standards at (200ng/ml), medium (600ng/ml) and high (1000ng/ml), and then compared with the true concentration of terbutaline. The accuracy of the bioanalytical method was greater than 99.4% (Table 6).

Stability

Stability studies should evaluate situations likely to be encountered during the sample processing, from

collection to final analysis. The stability of the urine standards was evaluated by assaying the analyte during sample collection, handling, after short-term storage and after going through freeze-and-thaw cycles. The stability of terbutaline in the urine matrix was examined by repeated injections (n=3) of the terbutaline urine samples that were stored at different temperatures (Table 7), and by comparing them with a freshly prepared standard.

A short-term stability test performed at room temperature showed that the samples were stable for 24 hr, and the mean recoveries were higher than 88.53%. Similarly, the long term stability study showed that terbutaline urine samples were stable for 1 month when stored at -20°C, with an average recovery **>**92.71%. No decrease in terbutaline concentration in urine was detected after exposing samples to three freeze/thaw cycles, and mean recovery ranged from 90.23 to 98.62% (Table 7).

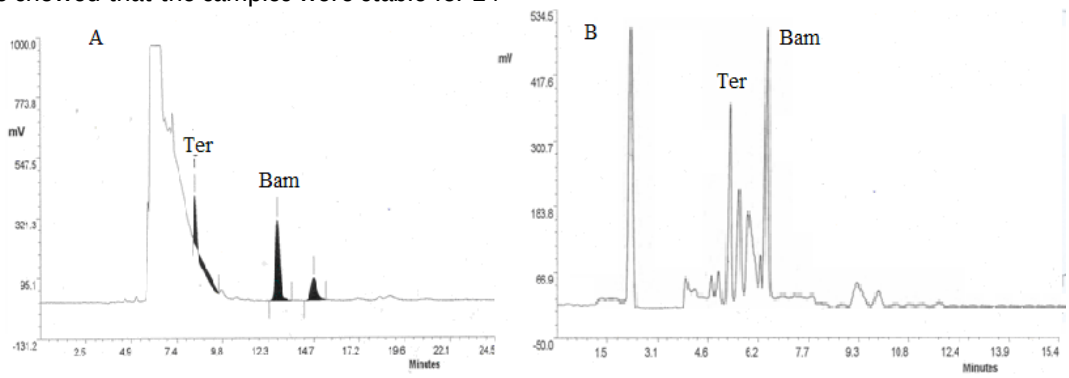

Figure 3: Comparison of MELC with other mobile phases i.e. A) methanol:OSA:phosphate buffer [20:1.1:78.9]. B) Micellar [Brij35 1.5%:butanol 2.5%:OSA 1.1%:buffer 94.9%] in urine.

Table 5: Intra-day and inter-day precision for terbutaline urine extraction method.

Nominal concentration (ng/ml)	Intra-day coefficient of variation (%)	Inter-day coefficient of variation (%)
200	4.21	2.89
600	3.56	4.28
1000	5.13	2.87

Table 6: Recovery and accuracy data for terbutaline spiked urine samples.

Actual concentration (ng/ml)	Observed concentration (ng/ml)	Recovery%	Accuracy%
200	201.7	94.02	100.8
600	596.4	87.64	99.4
1000	996.1	92.20	99.6

Table 7: Stability data of terbutaline spiked urine method.

Actual terbutaline Concentration (ng/ml)	Stored at room temperature for 24hr		Stored at -20°C for 1 month		Three freeze/thaw cycle	
	RSD %	Recovery %	RSD%	Recovery%	RSD %	Recovery%
200	3.49	**96.57**	4.83	**92.71**	3.97	**91.4**
600	4.35	**88.53**	6.74	**94.75**	2.63	**90.23**
1000	2.99	**100.49**	1.83	**99.16**	1.16	**98.62**

CONCLUSION

The method described in this study was precise and accurate and has equivalent sensitivity to conventional HPLC modes but with faster analysis time for the determination of terbutaline in biological fluid.

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