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Short communication

Extraction and determination of oxybutynin in human bladder samples by reversed-phase high-performance liquid chromatography

Renato Massoud[a,*], Giorgio Federici[a,b], Stefania Casciani[a,b], Savino M. Di Stasi[c], Giorgio Fucci[a], Antonella Giannantoni[d], Claudio Cortese[a]

[a]*Department of Internal Medicine, University of Rome "Tor Vergata", Via di Tor Vergata 135, 00133 Rome, Italy*
[b]*Children Hospital IRCCS "Bambino Gesù", Rome, Italy*
[c]*Department of Surgery, University of Rome "Tor Vergata", Rome, Italy*
[d]*"S. Lucia" IRRCS Rehabilitation Hospital, Rome, Italy*

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Abstract

A reversed-phase high-performance liquid chromatography method is described for the determination of oxybutynin (OXB) in human bladder samples. Following homogenization, tissue samples underwent double extraction with hexane and eventually were concentrated by freeze–drying before analysis. Chromatographic separation was performed with a mobile phase of acetonitrile–water–1 *M* ammonium acetate, pH 7.0 (85:13:2, v/v/v) at a flow-rate of 0.5 ml/min and double (electrochemical and UV) detection was applied. The retention time of oxybutynin eluting peak was around 18 min. Using a standard curve range of 10 to 500 ng/ml the quantification limit with electrochemical detection was 5 ng/ml with an injection volume of 100 μl. Within-day and day-to-day relative standard deviation values were 4.9 and 9.81%, respectively, while a 94% accuracy and a 72% recovery was attained. We applied this method to compare the OXB levels into bladder wall tissue samples after passive diffusion and after electromotive drug administration (EMDA), using a two-chambered poly(vinyl chloride) diffusion cell designed and developed in our laboratory. The results obtained show that EMDA enhanced OXB penetration into bladder wall and that this novel way of local drug administration can be potentially used in patients with neurogenic bladder dysfunction or urinary incontinence.

Keywords: Oxybutynin

1. Introduction

Oxybutynin (OXB; 4-diethylaminobut-2-ynyl 2-cyclohexyl-2-phenylglycolate) (Fig. 1) is an antispasmodic and anticholinergic drug used in the treatment of urinary bladder disorders by means of a direct relaxant effect on the bladder muscles [1,2].

Topical application of OXB by intravesical instillation is more effective than oral administration and is able to attain target tissue concentrations adequate for maximum clinical response [3]. Even if intravesical OXB instillation shows no toxicity, the clinical efficacy is similar to oral administration.

Some methods have been proposed for extraction and/or determination of oxybutynin in biological fluids [4] and pharmaceuticals [5], e.g., with gas chromatographic–mass spectrometric analysis or re-

*Corresponding author. Tel./fax: +39-06-7259-6204.
E-mail address: massoud@med.uniroma2.it (R. Massoud)

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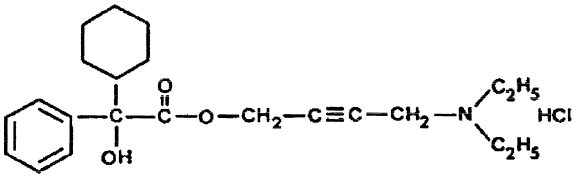

Fig. 1. Oxybutynin hydrochloride.

versed-phase ion-pair liquid chromatography, but no method is currently available to evaluate the drug concentration in tissue samples.

The object of this investigation was to develop a sensitive and selective method to quantify the levels of OXB in human bladder samples (e.g., after passive delivery or electromotive administration) for pharmacological and pharmacokinetic studies on neurogenic bladder [6], a pathological condition associated with incontinence.

It has recently been shown that a novel electromotive technique for drug administration [7] further enhances the delivery of OXB into the human bladder wall. In this study we have compared the OXB levels in the human bladder wall tissue after passive delivery (PD) with electromotive drug administration (EMDA). This latter methodology has recently been proposed for local controlled delivery of drugs [8]. Various electrokinetic phenomena can be recruited to accelerate drug administration across biological membranes and into underlying tissues. At least three such phenomena involved in electromotive transport are iontophoresis, electroosmosis/electrophoresis and electroporation.

We employed an appropriate apparatus consisting of a two-compartment poly(vinyl chloride) (PVC) diffusion cell, where viable tissue sample was placed in a frame with a central window of a fixed diameter sealed between the compartments of a diffusion cell and controlled conditions (oxybutynin and saline concentration, electric current, time) were applied. After each experiment the tissue sample was processed for the measurements of OXB.

2. Experimental

2.1. Chemicals

Oxybutynin-HCl (99%) was obtained from Sigma (St. Louis, MO, USA). All other chemicals were obtained from commercial sources and were, if needed, of HPLC-grade.

2.2. Apparatus

A System Gold Model 7.0 Beckman (Palo Alto, CA, USA) high-performance liquid chromatograph, equipped with a diode-array spectrophotometric detector (Model 168 Beckman) and an ESA (Chelmsford, MA, USA) 5100-A electrochemical detector was used. A C_{18} 4.6 μm particle size Ultrasphere ODS column (250 mm×4.5 mm) (Beckman) with guard column (45 mm×4.5 mm) packed with the same stationary phase was used.

2.3. Sample processing

From informed patients undergoing radical cystectomy for bladder cancer, a normal wall tissue section of approximately 2 cm^2 was obtained and the mass (g) recorded. Only patients who had not been assuming, in the previous two weeks, drugs potentially interfering with oxybutynin measurements were selected. The tissue sample was placed in cell culture medium at 4°C and transferred (time elapsed <30 min) to the laboratory for processing. After perivesical fat remotion the tissue specimen was exposed to an experimental model of either PD or EMDA of oxybutynin following the conditions recently described by us [9]. The samples after trimming were then homogenized for 1 min at room temperature in 0.1 *M* Tris–HCl buffer, pH 9.4, containing 10 m*M* 2,3-*tert*.-butyl-4-hydroxyanisole (BHA)–10 m*M* 2-*tert*.-butyl-4-methylphenol (BHT) (1:3, w/v) using a homogenizer Blendor (30-ml capacity) Model 7012F (Waring Products Division, USA). The homogenate was then centrifuged at 10 000 *g* for 20 min at 4°C. The resulting supernatant was mixed with acetonitrile in a ratio 1:0.5 (v/v) and extracted twice with hexane in a ratio 1:2 (v/v) for 10 min using glass tube and a horizontal shaker. The hexane layer obtained after centrifugation at 500 *g* for 5 min, was back-extracted into 0.1 *M* HCl (0.12 ml per ml of sample) by slight shaking for 10 min. After centrifugation at 500 *g* for 5 min the aqueous layer was freeze–dried and stored at −20°C until analysis. Before chromatography the sample was reconstituted with 200 μl of mobile phase for

high-performance liquid chromatography (HPLC). Results were expressed as ng/g of wet tissue.

2.4. Chromatography

The chromatographic conditions were as follows: mobile phase, acetonitrile–water–1 *M* ammonium acetate, pH 7.0 (85:13:2, v/v/v); flow-rate, 0.5 ml/min; detection potentials, 0.75 V and 0.85 V for analytical cell and 0.90 V for conditioning cell; detection wavelength, 235 nm; injection volume 50 μl; stop time, 30 min.

2.5. Calibration solutions

In order to obtain a suitable biological matrix a stock solution of oxybutynin in distilled water was prepared at a concentration of 5 mg/ml from which five calibration solutions were prepared by dilution with supernatant obtained from homogenate of drug-free human bladder, treated exactly as for experimental samples, at concentrations of 500, 100, 50, 25 and 10 ng/ml, respectively.

3. Results and discussion

Due to the high percentage of fibrous tissue that was present in human bladder samples, the process of homogenization was performed with a stainless steel blade assembled homogenizer. The procedures that utilize Ultra-Turrax or Potter apparatus were discarded because the homogenization products were incomplete.

Fig. 2B shows a typical HPLC chromatogram of a sample containing 140 ng/ml of oxybutynin obtained by organic extraction from a human bladder specimen; the retention time of OXB was around

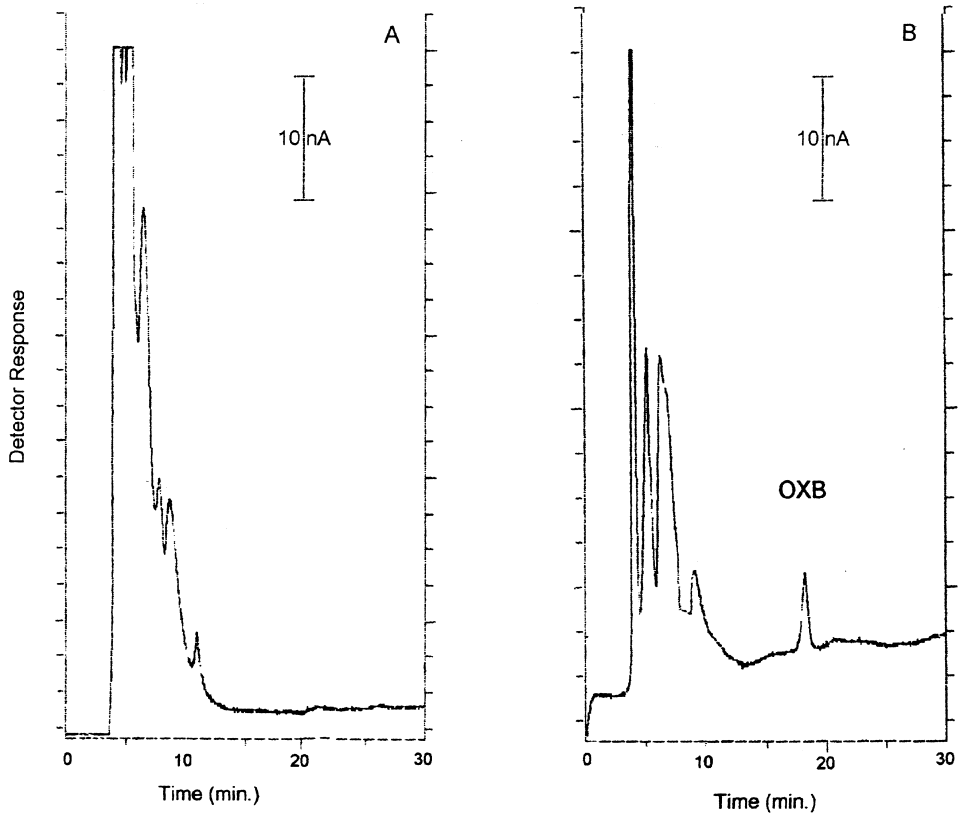

Fig. 2. (A) Chromatogram (blank) of a human bladder sample (supernatant of homogenate) obtained after organic extraction. (B) Chromatogram of a human bladder sample (supernatant of homogenate) containing 0.14 μg/ml (or 0.51 μg/g wet tissue) of oxybutynin.

18.00±0.4 min, whereas the endogenous components eluted before 15 min.

The UV spectrum analysis of eluting peak of OXB obtained by scanning with the diode array detector showed no evidence of coelution with other substances.

Each HPLC analysis was performed with both electrochemical and UV detection; in spite of a lower sensitivity the simultaneous UV 235 nm detection allows one to control for the efficiency of the electrochemical cells and reveal partial electrode deactivation events that are likely to occur when biological samples are derived from tissue extracts in which lipophilic materials are present. As a matter of fact it has appeared necessary to clean the graphite electrode of the analytical cell every ≈50 chromatographic analyses, while usually the working life of the electrode is longer when assaying other biological samples such as serum or urine.

Within-day precision was determined by analysis of seven different standard curves (range 10–500 ng/ml) on the same day using standard solutions of OXB hydrochloride prepared as described in Experimental. Day-to-day precision was determined by the analysis of the same solutions on 7 different days. The variability in the peak-area ratio at each concentration was used to determine the reproducibility of the assay procedure. The relative standard deviation values at concentrations of 10, 50, 100, 200 and 500 ng/ml for intra- and inter-day analysis were 6.9, 6.3, 4.8, 3.7, 1.9% and 15.4, 11.3, 7.1, 7.0, 5.4%, respectively.

To test the accuracy of the method, three control refrigerated samples (15, 35 and 70 ng/ml) were analyzed several times ($n=15$) during one week; the values obtained [(measured concentration/actual concentration)×100%] were 91.2, 93.8 and 97.9%, respectively. The relative standard deviation was less than 4.9%.

The recovery of oxybutynin from tissue samples (supernatants of homogenates) was assessed ($n=25$) at five concentration levels (10,50, 100, 200 and 500 ng/ml) by comparing the peak area after extraction with the peak area obtained from direct injection of equivalent quantities of unextracted reference standard. The mean recovery was 72.2% (43–105%) range for the five concentrations levels.

The regression equation of the calibration line obtained by plotting area under curve versus OXB concentration was: peak area=1.760×OXB concentration (ng/ml)−30.069, with coefficient of determination (r^2) of 0.980.

The lower quantification limit of this method defined as the amount which produces a signal equal to three-times the background noise levels was 5 ng/ml for 100 μl of extracted sample.

We have measured OXB tissue concentrations in twelve paired experiments on 24 bladder tissue samples from 12 patients undergoing radical cystectomy. Only tissue specimens free from any pathological process were used. Results in detail have been described elsewhere [7], briefly, urothelial areas of 0.78 cm^2 were exposed to the donor compartments and serosa to the receptor compartment in the two-chambered cell diffusion apparatus described above. In EMDA experiments the electrodes were connected to the current generator and experiments were performed with pulsed DC (2500 Hz) of 5 mA for 15 min. No electric current was applied in PD control experiments.

We were able to demonstrate that the mean quantities of OXB transported into the bladder wall samples by EMDA were significantly greater when compared to those recovered in PD experiments (8.69±4.52 μg vs. 2.02±1.75 μg; $p=0.0006$ by Wilcoxon signed rank test or 3.84±2.22 μg/g wet tissue vs. 0.87±0.78 μg/g; $p=0.0006$).

4. Conclusion

A simple and cost-effective HPLC method using a C_{18} column was developed for analysis of OXB in human bladder samples. In fact to avoid the use of gas chromatographic–mass spectrometric apparatus [10] and/or the use of particular columns such as CN or ion pair type columns [5] is economically advantageous. It is noteworthy that, although other methods described to measure the OXB concentration in biological fluids are able to give a slightly higher sensitivity, no methods are available for human tissue application. Furthermore the method described in this paper requires a simple liquid–liquid (hexane–supernatant homogenate) extraction procedure prior to the HPLC analysis with a reversed-phase

column. This method does not require an internal standard; furthermore with mean values of 4.7% inter-day and 9.2% intra-day variability and a sensitivity of 5 ng/ml, it seems suitable for pharmacological studies with human tissues. Comparable results (data not shown) have been obtained on serum and urine samples making it feasible to carry out complete pharmacokinetic studies.

We applied this method to compare the OXB levels into the bladder wall tissue samples after passive diffusion and after electromotive drug administration; the results obtained show that EMDA enhanced OXB penetration into bladder wall further enhancing the potential for local drug delivery thus avoiding systemic untoward side effects likely to occur in chronic administration. Further studies are needed in order to verify the use of EMDA in patients with neurogenic bladder and urinary incontinence.

References

[1] P.M. Lish, J.A. Labudde, E.L. Peters, S.I. Robbins, Arch. Int. Pharmacodyn. Ther. 156 (1965) 467–488.
[2] M. Tonini, C.A. Rizzi, E. Perucca, F. De Ponti, L. D'Angelo, A. Del Vecchio, A. Crema, J. Pharm. Pharmacol. 39 (1987) 103–107.
[3] C.B. Brendler, L.C. Radebaugh, J.L. Mohler, J. Urol. 141 (1989) 1350–1352.
[4] K.M. Hughes, J.T. Lang, R. Lazare, D. Gordon, S.L. Stanton, J. Malone-Lee, M. Geraint, Xenobiotica 22 (1992) 859–869.
[5] L.A. De Schutter, P. De Moerloose, J. Chromatogr. 450 (1988) 337–342.
[6] A.C. Dioko, J. Lapides, J. Urol. 108 (1972) 307–310.
[7] S.M. Di Stasi, A. Giannantoni, R. Massoud, C. Cortese, G. Vespasiani, F. Micali, J. Urol. 158 (1997) 228–233.
[8] M.A. Jewett, L. Valiquette, H.A. Sampson, J. Katz, Y. Fradet, D.A. Redelmeier, J. Urol. 161 (1999) 482–485.
[9] S.M. Di Stasi, G. Vespasiani, A. Giannantoni, R. Massoud, S. Dolci, F. Micali, Cancer Res. 57 (1997) 875–880.
[10] S.P. Kennerly, J.S. Markowits, J. Chromatogr. 487 (1989) 91–98.